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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Isao Karube et al.	Art Unit:	1636
Serial No.:	09/623,970	Examiner:	Lisa Gansheroff
Filed:	December 28, 2000		
Title:	SITE-SPECIFIC CELL PERFORATION TECHNIQUE		

Assistant Commissioner For Patents
Washington, D.C. 20231

DECLARATION OF TAKASHI SAITOH

I declare:

1. I am a co-inventor, along with Isao Karube, of the subject matter described and claimed in the above-referenced patent application or in any continuation or divisional application thereof.

2. I am a co-author on the Saito *et al.* (*Photochemistry and Photobiology* 68:745-748, 1998) publication. Any description of the invention described and claimed in the above-referenced patent application or in any continuation or divisional application thereof in this publication was the joint contribution of the present inventors alone, notwithstanding the inclusion of the additional authors, namely Nick A. Hartell, Hitoshi Muguruma, Shu Hotta, Satoshi Sasaki, Masao Ito, who were merely working under the direction of the present inventors providing technical assistance. These additional authors did not contribute to the conception and/or reduction to practice of the invention disclosed and claimed in the above-referenced application or in any continuation or divisional application thereof.

3. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 25 Oct. 2001

Takashi Saitoh

Part of Paper #11

PATENT

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Title:	SITE-SPECIFIC CELL PERFORATION TECHNIQUE		

Considered AMT 5/3/02

Assistant Commissioner For Patents
Washington, D.C. 20231

DECLARATION OF ISAO KARUBE

I declare:

1. I am a co-inventor, along with Takashi Saitoh, of the subject matter described and claimed in the above-referenced patent application or in any continuation or divisional application thereof.

2. I am a co-author on the Saito *et al.* (*Photochemistry and Photobiology* 68:745-748, 1998) publication. Any description of the invention described and claimed in the above-referenced patent application or in any continuation or divisional application thereof in this publication was the joint contribution of the present inventors alone, notwithstanding the inclusion of the additional authors, namely Nick A. Hartell, Hitoshi Muguruma, Shu Hotta, Satoshi Sasaki, Masao Ito, who were merely working under the direction of the present inventors providing technical assistance. These additional authors did not contribute to the conception and/or reduction to practice of the invention disclosed and claimed in the above-referenced application or in any continuation or divisional application thereof.

3. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: Nov. 6, 2001

Isao Karube

Isao Karube

Part of Paper #11

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Isao Karube et al.	Art Unit:	1636
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Considered AM 5/3/02

Assistant Commissioner For Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132

I, Katsuyoshi Ishii, do hereby make the following declarations:

1. I am a research scientist in the employ of the institute of physical and chemical research (RIKEN), Japan.

2. A copy of my curriculum vitae is attached as Exhibit 1.

3. I have reviewed the above-identified U.S. patent application (U.S. Serial No. 09/623,970, hereinafter "the '970 application") as originally filed as well as claims 1-5 as amended herewith.

4. I have reviewed the office action of July 12, 2001, particularly the rejection of claims 1-5 under 35 USC § 112, first paragraph, for lack of enablement.

5. On information and belief, claims 1-5 of the '970 application were rejected for failing to adequately teach how to make and/or use the invention, i.e., failing to provide an enabling disclosure. On information and belief, the Examiner stated the specification did not provide enablement for the full scope of combinations of compounds and specific stimulations to denature/perforate a membrane.

6. It is my opinion that one of ordinary skill in the art could identify operable combinations of "inducible membrane disrupting agent" and "stimulus" through routine experimentation.

7. For example, the initial efficacy of a particular combination can be judged immediately after treatment using an injection marker, such as a water-soluble fluorescent dye. If fluorescence is observed within the cell after treatment, then the treatment (i.e., the specific combination of reagent and stimulus) is judged to be successful. This process is described in the '970 application at Example 5, particularly at page 25, line 20 - 31. Thus, an injection marker can be routinely used to determine the efficiency of membrane disruption, and thereby identify effective combinations of "inducible membrane disrupting reagents" and "stimulus.

8. Likewise, the long-term efficacy of a particular combination can be judged using a conventional cell-detachment assay known in the art at the time of invention. By comparing the number of cells that detach from a culture dish a period of time after treatment (e.g., three to six days) to the number of initially cultured cells that remain on the culture dish immediately after the treatment, one of ordinary skill arrives at a detachment ratio. A particular treatment (i.e., a specific combination of reagent and

stimulus) is deemed suitable for practical use if the detachment ratio is less than the ratio of the control experiment. Thus, a conventional cell-detachment assay can be routinely used as a screening protocol to identify effective combinations of "inducible membrane disrupting reagents" and "stimulus".

9. The above described cell-detachment assay was published in the following references, copies of which are attached hereto as Exhibits A - D, respectively:

   - Reinhardt, CA et al. (1982) "Cell Detachment and Cloning Efficiency as Parameters for Cytotoxicity", *Toxicology*, **25**:47-52. See particularly, pages 48-49(MATERIAL AND METHODS), 51(Table I).
   - Altin JG, Wetts R Bradshaw RA (1991) Microinjection of a p21*ras* antibody into PC12 cells inhibits neurite outgrowth induced by nerve growth factor and basic fibroblast growth factor. Growth Factors **4:** 145-155. See particularly, pages146-151(Microinjection, Scoring Cells, Table 1 and RESULTS) ; and
   - Altin JG, Wetts R, Riabowol KT Bradshaw RA (1992) Testing the *in vivo* role of protein kinase C and c-Fos in neurite outgrowth by microinjection of antibodies into PC12 cells. Mol.Biol.Cell **3:** 323-333. See particularly, pages 324-325(MATERIAL AND METHODS, Table 1 and Table 2).
   - Atsumi T, Murata J, Kamiyanagi I, Fujisawa S. Ueha T. (1998 Jan.18.) Cytotoxicity of photosensitizers camphorquinone and 9-fluorenone with visible light irradiation on a human submandibular-duct cell line *in vitro*. Arch. Oral Biol. 43(1):73-81. See particularly, pages 75(Cytotoxity testing) and 78-79(Fig.5-7).

10. In light of the above, it is my opinion that one of ordinary skill in the art could make or use the invention commensurate with the scope of the claims from the guidance provided in the specification of the '970 application taken together with the teachings of the prior art.

11. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: Katsuyoshi Ishii
Katsuyoshi Ishii

Dated: December 10, 2001

Exhibit 1

CURRICULUM VITAE

Name, Family name: Ishii

Forename: Katsuyoshi

Sex: Male

Date of Birth: January 15, 1955

Place of Birth: Fukushima Prefecture, Japan

Nationality: Japanese

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Telephone +81-48-467-5928
Fax +81-48-467-5926

Educational, research, and professional experience:

1975 - 1981 School of Medicine, Yamagata University
Awarded the degree of M.D.

1981 - 1984 Obstetrician and Gynecologist worked Yamagata University hospital, Yamagata Prefectural hospital, and Sendai Red Cross hospital (including NICU).

1984 - 1988 Graduate School of Medicine, Yamagata University
Awarded the degree of Ph.D. in neuroanatomy for a thesis entitled "Postnatal development of the rat phrenic nucleus with Horse Radish Peroxidase(HRP) labeling method".
Worked supervised by Professor Toshio Shirai.

1988 -1998 Research Associate at the Department of Anatomy,
Yamagata University School of Medicine, under Professor T.Shirai.

During the 1995 -1996

A visiting researcher (A research worker abroad of the Ministry of Education, Science and Culture in Japan) at the Department of Anatomy and Neurobiology, University of California, Irvine, College of Medicine.

Worked supervised by Professor Edward G. Jones.

Studied the formation of connection between thalamus and cortex using intracellular injection of Lucifer Yellow in the brain slice.

1998.5. - present

Research Scientist at the Laboratory for Neural Architecture, Brain Science Institute (BSI), The Institute of Physical and Chemical research (RIKEN), Japan.

Membership of Academic Societies:

Anatomical Society of Japan.
Japan Neuroscience Society.
Society for Neuroscience of the United States of America.
International Brain Research Organization.

I have been studied the development of reticulospinal tract, corticospinal tract, corticocortical tract, and thalamocortical and corticothalamic tracts, using tract tracing methods(HRP, Wheat Germ Agglutinin combined HRP(WGA-HRP), DiI, Biocytin labeling methods) and an electron microscope. I have much experiences in microinjection method for labeling of cells.

Toxicology, 25 (1982) 47—52
Elsevier Scientific Publishers Ireland Ltd.

CELL DETACHMENT AND CLONING EFFICIENCY AS PARAMETERS FOR CYTOTOXICITY

CHRISTOPH A. REINHARDT, HANSPETER SCHAWALDER and GERHARD ZBINDEN

Institute of Toxicology, Swiss Federal Institute of Technology and University of Zurich, CH-8603 Schwerzenbach (Switzerland)

SUMMARY

Cell detachment and cloning efficiency of Baby Hamster Kidney cells (BHK-21 C13) were used as parameters to quantify cytotoxicity in vitro of 3 endogenous chemicals (glutathione, L-methionine, L-cysteine HCl) and 4 organotin compounds (tributyltinoxide, tributyltinchloride, tetrabutyltin, tetraphenyltin). IC_{50} values (inhibitory concentration at which the cloning efficiency was reduced to 50%) were estimated to be larger than 10^{-3} M for all 3 endogenous substances, which served as a calibration of the cell culture system for non-toxic chemicals. For the 2 tributyltin salts the IC_{50} values were estimated to be near 10^{-6} M and for the 2 tetraalkyltin compounds near 10^{-5} M.

The estimated CD_{50} values (concentration at which 50% of the cells detach) were at least twice as large as the corresponding IC_{50} values for all 7 chemicals tested. For the toxic tributyltin salts the cell detachment assay was 30—60 times less sensitive than the cloning efficiency assay. However, both assays rank all compounds tested in the same sequence of toxicity as that known from in vivo studies.

Key words: Quantitative cytotoxicity assay; Cell detachment; Cloning efficiency; Fibroblasts; Organotin compounds; In vitro

INTRODUCTION

Animal testing for acute and subacute toxicity of new industrial chemicals might be reduced if quantitative in vitro assays for cytotoxicity could be developed. Such assays should be sensitive, reproducible and should not give false negative answers. They must be validated with many classes of chemicals before it will be possible to judge their usefulness for a preliminary toxicological assessment of environmental chemicals and drugs.

In the present study, 2 independent parameters were used as indicators

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for cytotoxicity, i.e. cell detachment and cloning efficiency. Both parameters cover a wide variety of cellular damage. Cell detachment covers an irreversible damage to the cytoskeleton during a 4-h period. Cloning efficiency covers influences on growth of single cells during a 6-day period, and includes cell reattachment followed by clone formation.

Two groups of substances were chosen on the basis of their toxicity and their chemical structure. (1) Three endogenous compounds served to pinpoint the effective concentration range of the cell system for non-toxic chemicals. (2) Four moderately toxic organotin compounds were tested in order to detect a correlation between their toxicity in vivo [7] and the cytotoxicity in vitro.

MATERIALS AND METHODS

The established cell line BHK-21 C13 was kindly provided by Dr. G. Mindek (Zurich) and was grown at subconfluent conditions (subculture 1:5 to 1:10 every 3—4 days) in Eagle's minimum essential medium (MEM) including 10% fetal calf serum and 1.5 g/l $NaHCO_3$ (all substances for media from Böhringer/Mannheim), in 5% CO_2 plus air. A large cell batch (5×10^7 cells) served as a stock culture which was divided into test batches ($1—2 \times 10^6$ cells), then frozen in 1 ml cryotubes (Nunc) and stored in liquid nitrogen until used (in MEM plus 10% DMSO). Two to ten days before the experiment, a frozen cell batch was thawed and subcultured up to 3 times.

The endogenous compounds (glutathione, L-methionine, L-cysteine HCl; crystalline puriss., Serva) were diluted in the medium just before the incubation was started. The organotin compounds (tributyltinoxide, tributyltinchloride, tetrabutyltin, tetraphenyltin) were kindly provided by Acima AG (Buchs) in the form used for industrial production. They were dissolved in acetone and pH-adjusted (with 1 M NaOH to pH 7.2) in a 10% mixture with MEM ("test solution"). In case of the tetraalkyltin compounds, the highest concentrations tested (10^{-5} M for tetraphenyltin, 10^{-4} M for tetrabutyltin) reached the limits of solubility in the culture medium and could not be tested at higher concentrations.

Cell detachment was quantified with a Coulter Counter (Model ZBI, Coulter Electronics, Herts, England) combined with a channelizer and plotter. In order to determine the optimal counting conditions, channelizer and plotter were used to monitor the cell size distribution of normal (freshly trypsinized) cells and of detached (experimental) cells. The following conditions on the Coulter Counter were selected: window width = 100, base channel threshold = 3, aperture current = 8, amplification = 32. For each cell detachment assay 20 000 (equal to 10^4 cells/cm^2) cells were plated in each well of a 24-well plate (Nunc), and allowed to settle and attach during 24 h. A constant volume of medium (0.45 ml MEM) was supplied to each well with 0.05 ml of test solution. The controls for the organotin compounds received a final concentration of acetone of 1%. After a 4-h incubation the detached cells were removed from each well by adding 1 ml of

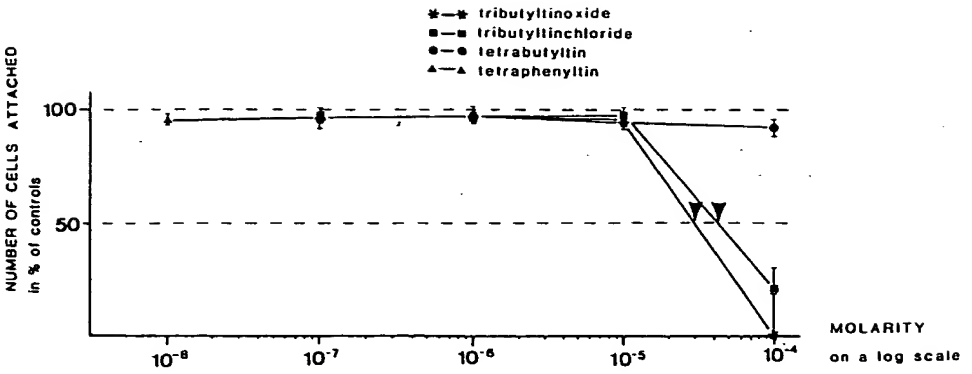

Fig. 1. Mean and standard deviation of 4 parallel experiments for the cell detachment assay (BHK-21 C13 cells after 4 h incubation with the test compound). Detached cells were counted and the value subtracted from the total number (20 000) of cells/well. Glutathione, L-methionine and L-cysteine HCl are not included since their values (including the experiment for the highest concentration of 5×10^{-3} M) were not significantly different from controls (see Table I). The arrowheads indicate where the values for CD_{50} (concentration where 50% of the cells detach from the culture dish) were read.

isoton II (Coulter Electronics), pipetted twice, transferred into 5 ml cuvettes (final dilution 1:10) and counted in the Coulter Counter with a 50-μm orifice. The number of detached cells was subtracted from the total number of cells plated and the mean and standard deviation of 4 parallel experiments were calculated. The values were plotted on logarithmic graph paper and the concentration at which 50% of the cells detached (CD_{50}) was estimated as shown in Fig. 1. The remaining cells in the plate were fixed and stained with Giemsa in order to check for morphological alterations of the cells.

Cloning efficiency of the BHK-21 C13 cells was determined in 24-well plates with the identical mixture of culture medium and test solution as used in the cell detachment assay. Each well received 100 BHK cells immediately before the test solution was added. No medium change was made during the whole assay. After 6 days the cells were fixed and stained with methylene blue or Giemsa. The number of cell colonies formed (more than 20 cells = 1 colony) were counted under a dissecting microscope (12 X magnification), and the mean and standard deviation of 4 parallel experiments were calculated. The control value (MEM with and without 1% acetone) was determined in each experiment (cloning efficiency varied between 35% and 40% of the total cell number plated), and the experimental value was expressed as percentage of that control. The inhibitory concentration at which the cloning efficiency was reduced to 50% of the control (IC_{50}) was estimated as shown in Fig. 2.

RESULTS AND DISCUSSION

All test compounds were simultaneously tested in both assays, on 2 differ-

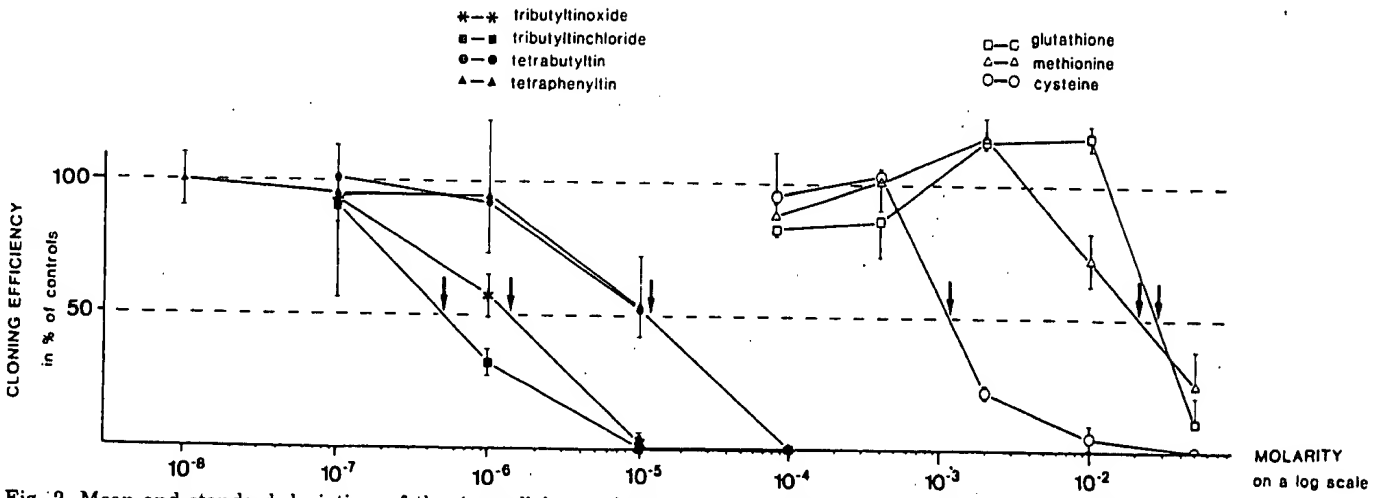

Fig. 2. Mean and standard deviation of the 4 parallel experiments from the cloning efficiency assay (BHK-21 C13 cells after 6 days of growth with the test substance present from plating on). The number of cell clones formed was counted and expressed as percentage of the controls. The arrows indicate where the values for IC_{50} (inhibitory concentration to 50% of the control level) were read.

TABLE I

SUMMARY OF THE RESULTS FROM THE CELL DETACHMENT ASSAY (4 H IN MEM) AND THE CLONING EFFICIENCY ASSAY (6 DAYS IN MEM) WITH BABY HAMSTER KIDNEY CELLS (BHK-21 C13) IN VITRO

Chemical	CD_{50}		IC_{50}		LD_{50} (mg/kg) (rat)
	molar	ppm[a]	molar	ppm[a]	
Glutathione	$>5 \times 10^{-3}$	15×10^{3}	2.3×10^{-3}	7×10^{3}	not listed
L-Methionine	$>5 \times 10^{-3}$	8×10^{3}	2.1×10^{-3}	3×10^{3}	4300 [2]
L-Cysteine HCl	$>5 \times 10^{-3}$	9×10^{3}	1.2×10^{-3}	2×10^{2}	not listed
Tributyltinoxide	3.0×10^{-5}	18	5.0×10^{-7}	0.3	148-234 [1]
Tributyltinchloride	4.3×10^{-5}	14	1.4×10^{-6}	0.5	122-350 [1]
Tetrabutyltin	$>10^{-4}$	>35[b]	1.2×10^{-5}	4.1	>4000 [1]
Tetraphenyltin	$>10^{-5}$	>4[b]	1.2×10^{-5}	5.1	not listed

[a] In μg/ml culture medium.
[b] Limit of solubility in culture medium.
CD_{50} = concentration at which 50% of the cells detach within 4 h.
IC_{50} = concentration at which cell clone formation is inhibited by 50%.

ent 24-well plates. This allowed us to calibrate the sensitivity of the 2 assay procedures relative to each other as well as to detect differences in the effects between the various chemicals. Besides the easy handling of the 24-well plates they offer the advantage of low costs due to the small volume of medium necessary per well. Cell detachment from the plastic dish was irreversible after the 4-h period of our assay. The detached cells were always smaller than the viable cells which indicates a probably lethal loss of cytoplasmic volume as a result of the toxic action of the tested compound.

For all 7 test chemicals, the detachment assay was more than twice, and for the 2 tributyltin salts 30—60 times less sensitive than the cloning efficiency assay (Table I). The reason for this difference may not only lie in the different incubation times (4 h vs. 6 days) but also in the different cell functions involved. Cell detachment from a tightly attached monolayer of cells involves a drastic destruction of the cytoskeleton, whereas cloning efficiency is influenced when parameters, such as uptake of nutrients, protein synthesis and plating efficiency, are affected. The effects of xenobiotics on the plating efficiency during reattachment of trypsinized cells needs further investigation, which will allow us to distinguish it from more relevant growth parameters measured by the cloning efficiency assay (work in progress).

Glutathione, L-methionine and L-cysteine HCl were used to calibrate the system in order to find the effective concentration range for non-toxic chemicals. For all 3 compounds the highest concentration of 5×10^{-2} M had no effect on either detachment or the morphology of the BHK cells (see Table I). In the cloning efficiency assay the IC_{50} values were between

10^{-3} and 3×10^{-2} M (Fig. 2, Table I). For this estimation, the amount of methionine and cysteine added to MEM was used.

For the organotin compounds, the toxic concentration range in the cloning efficiency assay was equal to or higher than 10^{-6} M for the 2 tributyltin salts and equal to or higher than 10^{-5} M for the 2 tetraalkyltin compounds (Fig. 2). As clarified by a conversion of molar values to ppm values (Table I) the difference between the concentration ranges of organotin compounds compared to the endogenous substances is in the order of 1000 times. The tetraalkyltin compounds showed no effect in the cell attachment assay at all concentrations (Fig. 1). Both of our in vitro assays ranked the tributyltin salts as more toxic than the tetraalkyltin compounds. These results correlate well with in vivo (LD_{50}) studies in rats (see Table I). Experiments with whole animals have generally revealed a much higher toxicity for trialkyltin salts than for tetraalkyltin compounds [2,3,4].

The results reported in this paper show that our cell culture assay can probably serve as a simple, rapid and quantitative system with which the cytotoxic potential of chemicals can be evaluated. Our selected fibroblast cell line (BHK-21 C13) is easily available, grows rapidly and has quantifiable cell detachment properties. Other cell strains need not necessarily give the same results with the 2 assays described, although it is anticipated that other cell types need to be tested in order to establish the reliability and validity of an in vitro cytotoxicity test. As a prescreen assay for cytotoxicity, such a test could ultimately serve as an alternative to animal testing in the general sense of producing new and different information on the hazard of environmental chemicals and might also contribute to the reduction of the number of laboratory animals.

ACKNOWLEDGEMENTS

This project was supported by the "Schweiz. Tierschutz" and the "Fonds für versuchstierfreie Forschung" (Zurich). We would like to thank Drs. B. Carden, P. Maier and G. Moser for their critical comments to the manuscript.

REFERENCES

1 P.J. Smith, Toxicological data on organotin compounds. In: Int. Tin Res. Inst., Publ. 538 (1977) 1.
2 Register of Toxic Effects of Chemical Substances NIOSH, Washington, Publ. 78104 A (1977).
3 R.S. Dyer, T.J. Walsh, H.S. Swartzwelder and M.J. Wayner, (Eds.), Neurobehavior, Toxicol. Teratol., 4 (2) (1982).
4 World Health Organisation, Tin and organotin compounds. A preliminary report. WHO report 15 (1981).

Growth Factors, 1991, Vol. 4, pp. 145–155
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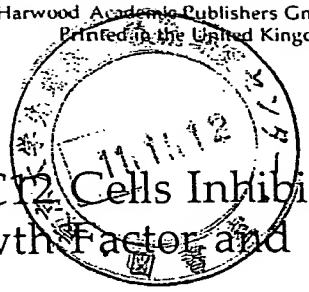

Microinjection of a p21*ras* Antibody into PC12 Cells Inhibits Neurite Outgrowth Induced by Nerve Growth Factor and Basic Fibroblast Growth Factor

JOSEPH G. ALTIN[1†], RICHARD WETTS[2] and RALPH A. BRADSHAW[1*]

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(Received 18 April 1990, Accepted 15 August 1990)

The role of p21*ras* in signal transduction in PC12 cells was studied using an antibody that blocks its function. Native cells were microinjected with either a control solution or a solution containing the monoclonal antibody Y13-259. Treatment of the cells with growth factors appeared to enhance the ability of the cells to survive the microinjection procedure. Of the cells microinjected with the control solution 66–69% of those treated with either nerve growth factor (NGF) or basic fibroblast growth factor (bFGF) were still present 24 h postinjection, compared with only 57% for those not treated with growth factor after microinjection. This effect of the growth factors was inhibited by introduction of the Y13-259 antibody, suggesting that it occurs through a pathway that involves p21*ras*. Similarly, introduction of the Y13-259 antibody into cells also resulted in a statistically significant decrease in the percentage of neurite-bearing cells; 25–36% of the cells microinjected with the control solution had neurites, whereas 12–14% of the cells microinjected with the antibody solution had neurites. This decrease suggests that the induction of neurite outgrowth and the maintenance of established neurites by these growth factors is dependent on a functional p21*ras* pathway. As well as complementing the finding that p21*ras* is apparently involved in the mechanism of action of NGF in PC12 cells, these results further establish (1) that p21*ras* is also involved in the mechanism of action of bFGF, and (2) that the effect of NGF and bFGF on the number of labeled cells still present 24 h postinjection requires a functional p21*ras* protein.

KEYWORDS: PC12 cells, NGF, bFGF, p21*ras*, neurite outgrowth, cell survival

INTRODUCTION

Differentiation and survival of many cell types depends on extrinsic growth factors, which mediate their effects through a variety of second messenger systems. A widely used model system for studying the signal transduction pathway(s) leading to neuronal differentiation is the rat pheochromocytoma cell line PC12 (Greene and Tischler, 1982). In these cells, either nerve growth factor (NGF) or basic fibroblast growth factor (bFGF) can induce a differentiation of the cells from a round chromaffin cell-like morphology into one that resembles sympathetic neurons (Greene and Tischler, 1982; Togari et al., 1985; Schubert, Jing and Baird, 1987; Rydel and Greene, 1987). The molecular mechanisms underlying the morphological and biochemical changes induced by these neurotrophic factors are not yet fully understood.

Recent evidence suggests that in certain cells the p21*ras* family of proteins may play a role in signal transduction (Barbacid, 1987; Nishimura and Sekiya, 1987; Santos and Nebreda, 1989). The proteins in this family are homologous, with molecular weights of ~21 kDa, and possess an intrinsic GTPase

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activity. The microinjection of p21*ras* into NIH 3T3 cells leads to their transformation (Stacey and Kung, 1984), whereas the microinjection of an antibody to p21*ras* (specifically monoclonal antibody Y13-259; Furth et al., 1982) can induce a reversal of the transformed phenotype in these cells (Kung et al., 1986). In addition, microinjection of this antibody can inhibit the initiation of DNA synthesis by serum in NIH 3T3 cells, the proliferation of NIH 3T3 cells transformed by receptor-like oncogenes possessing tyrosine kinase activity (Mulcahy, Smith and Stacey, 1985), and the proliferation of certain types of tumor cells (Stacey, DeGudicibus and Smith, 1987). These findings suggest a role for p21*ras* in the control of cellular proliferation.

Other experiments suggest that p21*ras* is involved in the differentiation of neuronal cells. Microinjection of the oncogenic Ha-p21*ras* (Bar-Sagi and Feramisco, 1985), or of the protooncogenic Ha-*ras* protein preincubated with guanosine-5'-O-3-thiotriphosphate (Satoh, Nakamura and Kaziro, 1987), into PC12 cells induces morphological differentiation similar to that induced by NGF (and bFGF). Similarly, morphological differentiation is observed following transfection of the activated N-*ras* gene into PC12 cells (Guerrero et al., 1986), and the introduction of *ras* proteins into cultured embryonic neurons mimics the biological activity of neurotrophic factors in vitro (Borasio et al., 1989).

The dual role of p21*ras*, appearing to be involved in inducing proliferation in some cell types (for example, NIH 3T3 cells), but differentiation in others (for example, PC12 cells), highlights the importance of understanding the precise role of this protein in the signaling cascades. In a previous attempt to investigate the involvement of p21*ras* in NGF-induced differentiation, the antibody Y13-259 was microinjected into fused cells (Hagag, Halegoua and Viola, 1986). These cells apparently possess many normal characteristics, including their ability to extend neurites in response to NGF, and their ten-fold increase in size over native PC12 cells greatly facilitates microinjection procedures. However, such fused cells are multinucleated, are incapable of undergoing cell division, and may possess differences that limit the interpretation of the results. In this work, we have circumvented this potential limitation by microinjecting the monoclonal antibody Y13-259 directly into native (unfused) PC12 cells. The results show an involvement of p21*ras* in both bFGF-induced and NGF-induced neurite outgrowth in PC12 cells.

METHODS

Materials

Monoclonal antibody Y13-259 was a gift from Dr Jackson B. Gibbs, Merck Research Laboratories (Rahway, New Jersey). A lyophilized form of the antibody supplied by Oncogene Sciences Inc. was found to be equally effective in inhibiting neurite outgrowth induced by NGF and bFGF (data not shown). NGF was prepared as β-NGF by the method of Mobley, Schenker and Shooter (1976). A bFGF analog in which all half-cystine residues were replaced by serines (Fox et al., 1988), was kindly provided by Dr Gary M. Fox, Amgen Inc. (Thousand Oaks, California). This analog was found to be indistinguishable from preparations of the natural sequence in stimulating neurite outgrowth and phospholipid metabolism in PC12 cells (Altin and Bradshaw, 1990).

Cell Culture

PC12 cells were adapted to grow in Dulbecco's modified Eagle's medium (DME, Flow Laboratories) supplemented with 10% fetal calf serum (Irvine Scientific), 5% heat-inactivated horse serum (Cell Culture Laboratories) and 1% Fungi-Bact solution (Irvine Scientific). Stock cells were grown in Belco T150 tissue culture flasks and were maintained at 37°C in a humidified atmosphere of 5% CO_2; the medium was changed every 2–3 days, and the cells were passed weekly.

Microinjection

Cells for microinjection were seeded in collagen-coated (Greene and Tischler, 1982) six-well plates (Limbro) at a density of around 4×10 cells/cm^2 in low serum media (DME supplemented with 2% fetal calf serum and 1% heat-inactivated horse serum) in the presence of either NGF (100 ng/ml) or bFGF (5 ng/ml) for 48 h before microinjection. To buffer the medium while the cells were out of the incubator, HEPES (20 mM, pH 7.4) was added to the cells following their removal from the incubator. Immediately before microinjection the medium was replaced with warmed Dulbecco's phosphate-buffered saline (pH 7.4), which also contained 10 mM glucose. After microinjection the cells were kept in this solution for 1–2 h at 37°C before replacing with low serum media (as above) in the

presence of the same growth factor as for the previous 48 h.

Individual cells were microinjected with either a control solution or an antibody solution. The control solution was composed of 60 mM KCl, 10 mM NaCl, and 0.1 mM EDTA, and the antibody solution was control solution plus Y13-259 antibody (8 mg/ml). The use of a control solution which contained also 8 mg/ml of bovine serum albumin (Fraction V, Sigma), or an equivalent amount of Y13-259 antibody that was inactivated by excessive freeze-thawing, gave results that were indistinguishable from those obtained using a solution in which the bovine serum albumin or the inactivated antibody was omitted (Table 1). To mark the injection cells, rhodamine-conjugated 70 kDa dextran (100 mg/ml, no. D-1818, Molecular Probes) was present in both the control and the antibody solution. The micropipettes were pulled from filament glass tubing (1.2 mm/0.9 mm, American Medical Systems) on a Flaming-Brown P-80/PC puller (Sutter Instruments). The injected solution was expelled with air pressure controlled by a PicoSpritzer II (General Valve Corp). The amount of solution expelled was variable, but we attempted to inject approximately 10% of the cell volume. To visualize the injected cells, the microinjections were performed using an epifluorescence, fixed-stage microscope (Lab 16, Zeiss) equipped with a long working distance 40 × objective (Nikon). After microinjection of a group of cells, the total number of rhodamine-labeled cells was counted; any cells (healthy or not) that detached immediately were therefore not included in the count.

Scoring Cells

Rhodamine-labeled cells were scored 24 h after microinjection using the 40 × objective on an epifluorescence microscope (Universal, Zeiss) equipped with a light-intensifying SIT video camera (RCA) and an image processor (151, Imaging Technologies). As the cells were being scored, video images were stored on either videotape (U-matic, Sony) or videodisc (TQ-2028F, Panasonic). The cells were scored into one of three groups as described in the Results section.

Immunocytochemistry

To determine how long the rat monoclonal antibody Y13-259 remained in the PC12 cells, immunocytochemistry was performed at 24, 32, and 48 h after microinjection. The cells were rinsed with phosphate buffer (0.1 M, pH 7.4) and fixed with 4% paraformaldehyde in phosphate buffer (30 min at room temperature). After three rinses in this buffer, the cells were permeabilized and blocked with a solution of 20% goat serum plus 0.2% Triton X-100 in phosphate buffer (1 h), followed by incubation with FITC-conjugated anti-rat IgG (Zymed) diluted 1:20 in the same solution (1.5 h at room temperature). Following washing with this solution (0.5 h) and with the phosphate buffer, rhodamine and fluorescein fluorescence was visualized to detect the presence of the dextran, and the Y13-259 antibody, respectively, with the same equipment used to score the cells.

TABLE 1
The Percentage of Injected Cells in Each of Four Classes 24 h After Microinjection with Either Anti-p21*ras* Antibody or with Control Solution

Condition		Cells (%)				Total cells injected	Number of experiments
		Neurites	Round	Abnormal	Missing		
NGF	Control	36	14	19	31	180	(6)
	Y13-259**	12^{aa}	20	23	46^{a}	160	(6)
bFGF	Control	25	25	16	34	359	(10)
	Y13-259**	14^{aa}	22	21	43^{a}	349	(11)
No GF	Control	8	30	19	43	145	(6)
	Y13-259	7	28	17	48	159	(6)
NGF	Control	33	17	12	38	224	(7)
	Inactive Ab	25	17	11	48	204	(6)

The 4-by-2 chi-square test for equality of 2 multinomials was used to compare all four categories simultaneously. There was a significant difference between the Y13-259-injected cells and control-injected cells for both the NGF- and bFGF-treated cells (**$p < 0.001$). Upon finding a difference with the chi-square test, the unequal pairs were identified by the Irwin–Fischer test, using the normal approximation to the hypergeometric distribution ($^{a}p < 0.01$; $^{aa}p < 0.001$).

When no growth factor was present after microinjection ('no GF' condition) or when the antibody was inactive ('inactive Ab'), there were no significant differences between control-injected and antibody-injected cells.

RESULTS

Microinjection of Monoclonal Antibody Y13-259 into PC12 Cells

Microinjections were performed on PC12 cells that had been pretreated with NGF or bFGF for 48 h. A significant proportion of these cells (50%) possessed neurites at the time of microinjection, while the remainder were either slightly flattened or showed little if any response. The formation and maintenance of neurites is an effect of these growth factors, because the percentage of cells that spontaneously grow neurites in the absence of the growth factor is very low (<1%). Only the neurite-bearing cells were microinjected, because their viability was much better than non-responding cells (data not shown), and because they maintained or regrew their neurites within 24 h after microinjection. Individual cells were microinjected with either a control solution or an antibody solution; both solutions contained rhodamine dextran to permit identification of the injected cells (Wetts and Fraser, 1989). The co-injection of the solution containing both the dye and the rat monoclonal antibody Y13-259 resulted in the successful introduction of both into the cell; the antibody was detectable by immunocytochemistry using a fluorescein-conjugated anti-rat IgG antibody. After 24 h, ~80% of the rhodamine-labeled cells were also fluorescein-labeled (Fig. 1a, b). Even after 48 h, half of the cells had detectable levels of Y13-259 (data not shown).

Rhodamine-labeled cells, examined 24 h after microinjection, were scored blind to the injected solution (either control or antibody-containing). The number of very faint cells (that is, those which contained small amounts of rhodamine and antibody) was very low (1–2%) for both control and antibody injections. Each clearly labeled cell was classified into one of three groups: neurites, round, or abnormal. Cells were scored as responsive ('neurites') if they had processes of at least one cell diameter in length or had multiple neurites. 'Round' cells had small or no neurites. Round and neurite cells appeared to be healthy. Other cells were more adversely affected by the microinjection procedure and were scored as 'abnormal'. These cells were either very faint or exhibited a punctated appearance under fluorescence. Under bright-field illumination, the cells were smaller, darker, and more granular than the healthy cells. To determine the number of 'missing' cells, the total number of labeled cells in these three groups was subtracted from the number of cells injected on the previous day. These 'missing' cells presumably had died and/or became detached and were lost in changing the medium. The percentages of cells of these four categories is presented in Table 1.

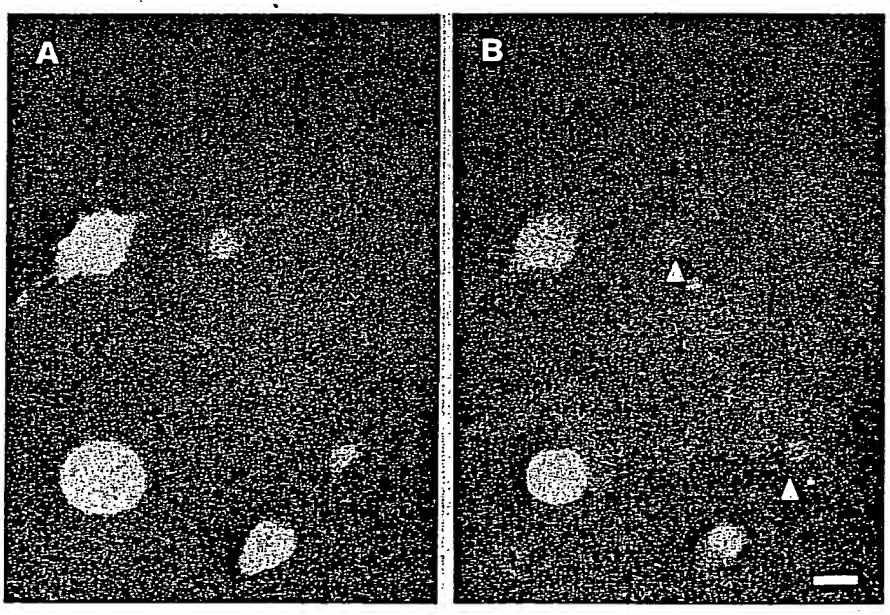

FIGURE 1. Rhodamine fluorescence (A) of cells 24 h after microinjection with a solution containing rhodamine dextran and Y13-259 antibody. Fluorescein fluorescence (B) shows the presence of Y13-259 in the same cells following immunocytochemistry with fluorescein-conjugated anti-rat IgG antibody. Uninjected cells have high background levels of fluorescence, possibly due to crossreaction between the anti-rat IgG antibody and the rat PC12 cells. Two cells were labeled with a small amount of rhodamine (A) and apparently had low levels of Y13-259 antibody (arrowhead in B). Brightly labeled cells appear round (for example, the two cells near the bottom), because bright fluorescence causes 'blooming' in the SIT videocamera. Scale bar = 20 microns (μm).

Effect of Y13-259 Antibody on Cell Number

After microinjection of the control solution, the number of injected cells that were considered to be normal and healthy was around 50% (Tables 1 and 2). This result was similar for both NGF-treated and bFGF-treated cells. As shown in Table 1, a significant fraction of the cells (16–19%) appeared abnormal; many of these may have suffered irreversible damage from the injection trauma. The remaining 31–34% of injected cells were 'missing' at the time of scoring; some of these may not have survived the microinjection. Many of the cells considered to be healthy were still capable of extending long neurites (Fig. 2a, b), which suggests that the microinjection trauma or the presence of the rhodamine did not grossly affect the action of the growth factors in these cells.

TABLE 2
The Percentage of PC12 Cells that Were Healthy, and the Percentage of Healthy Cells that Had Neurites 24 h after Microinjection

Condition		Cells (% healthy)	Healthy with neurites	Number of experiments
NGF	Control	50	72	(6)
	Y13-259	32**	37**	(6)
bFGF	Control	50	50	(10)
	Y13-259	36**	39*	(11)
No GF	Control	38	20†	(6)
	Y13-259	35	20†	(6)
NGF	Control	50	67	(7)
	Inactive Ab	42	60	(6)

Percentages for antibody-injected cells were significantly different from control-injected cells by 2-by-2 chi-square test (**$p < 0.001$; *$p < 0.05$).

When no growth factor was present after microinjection ('no GF' condition) or when the antibody was inactive ('inactive Ab'), there were no significant differences between control-injected and antibody-injected cells. For the 'no GF' condition, these percentages were significantly different from the percentages for growth factor-treated, Y13-259-injected cells by 2-by-2 chi-square test (†$p < 0.003$).

To determine whether the growth factor played a role in the number of microinjected cells present after 24 h, we conducted experiments in which bFGF was omitted for the 24 h postinjection. Under these conditions, the percentage of cells that were scored as missing following microinjection with the control solution was increased to 43% (see Table 1). This result is significantly different from the percentage of missing cells that is obtained when either NGF or bFGF is present for the 24 h postinjection period (31–34%), indicating that the addition of growth factor does prevent the loss of cells after impalement.

By comparison with the control solution, microinjection of the Y13-259 antibody resulted in a significant increase in the number of cells that were missing 24 h postinjection (see Table 1). This effect was observed for cells microinjected with either NGF or bFGF; the proportion of cells that were missing at the time of scoring was 46% and 43%, respectively. These percentages are similar to those obtained from microinjections (of either antibody or control solution) into cells that were not treated with growth factor during the 24 h postinjection (43–48%, see Table 1). The data in Fig. 3(a) shows the percentages of missing cells that were scored for each independent experiment. It can be seen that microinjection of the antibody was associated with a greater number of points for which the percentage of missing cells was higher than the control. Further, if the antibody solution is partially inactivated by freeze-thawing, then the number of missing cells is not significantly different from the number obtained from contemporaneous injections of the control solution (see Table 1). Thus, the introduction of the Y13-259 antibody by microinjection into PC12 cells affects the number of missing cells to the same degree as removing NGF or bFGF.

Effect of Y13-259 on Neurite Outgrowth

Interestingly, microinjection of the control solution causes neurite retraction in a proportion of microinjected cells (see Tables 1 and 2). Although all cells had neurites at the time of microinjection, only 72% of the healthy, NGF-treated cells had neurites when scored 24 h after injection. This effect was even more pronounced for cells treated with bFGF; microinjection of the control solution resulted in a reduction in the number of neurite-bearing cells to ~50% (see Table 2). Healthy cells with neurites either maintained their neurites, or first retracted and then regrew them, during the 24 h after microinjection. Since less than 100% of the cells had neurites, and there is no evidence that uninjected NGF-treated or bFGF-treated cells spontaneously withdraw their neurites, the results suggest that the trauma of the microinjection caused neurite retraction in a significant proportion of the cells.

The microinjection of the Y13-259 antibody into PC12 cells resulted in a further reduction in the number of neurite-bearing cells compared to their respective controls (see Tables 1 and 2). Of the 180 NGF-treated cells that were microinjected with the control solution, 36% were scored as having

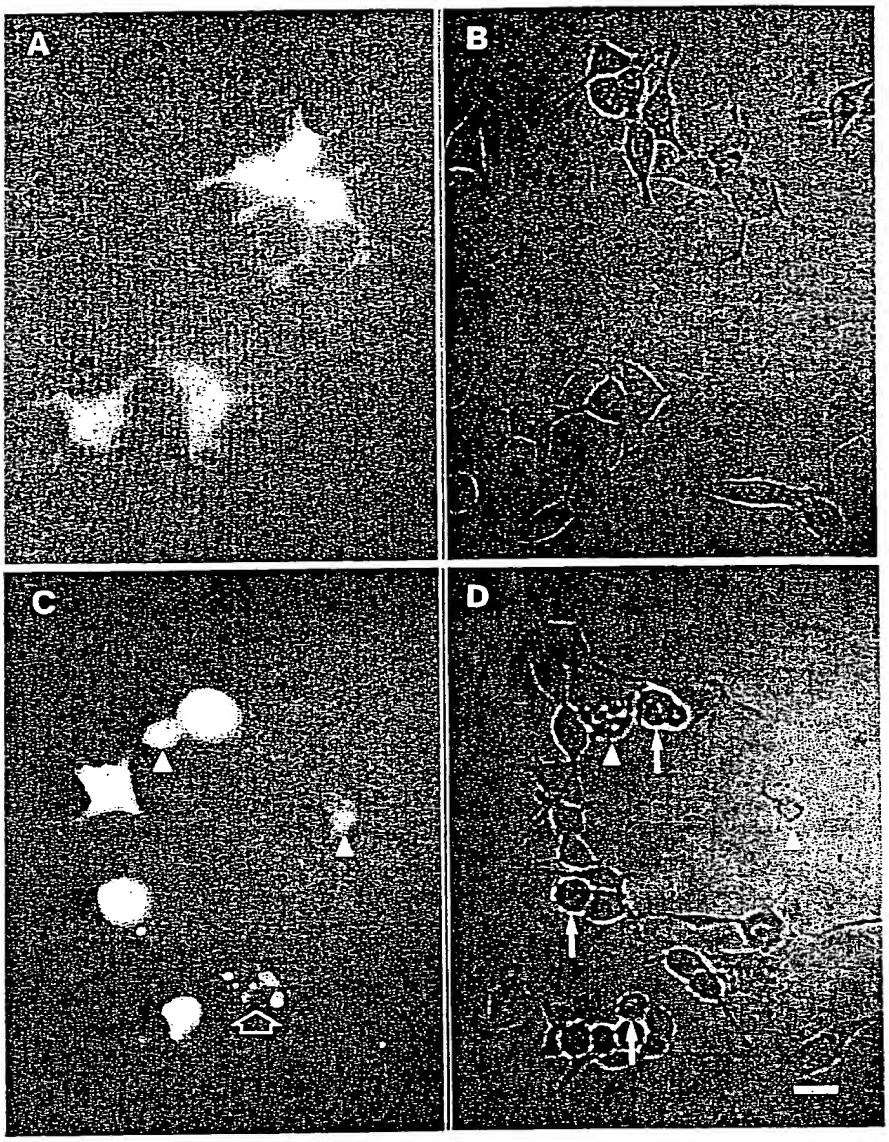

FIGURE 2. Effect of microinjection of Y13-259 antibody into PC12 cells. PC12 cells treated with bFGF were microinjected with either a control solution (A, B) or a solution containing Y13-259 antibody (C, D). After 24 h the microinjected cells were identified with rhodamine fluorescence (A, C). The five labeled cells shown in A were injected with control solution; these cells have neurites and appear indistinguishable from uninjected cells in the bright-field photograph (B). The cells shown in C were injected with antibody solution; in bright-field (D) two cells appeared abnormal (arrowheads), three were round (arrows), and one had short neurites. Fluorescent debris is marked by the open arrow and does not indicate a microinjected cell. Scale bar = 20 microns (μm).

neurites 24 h after microinjection (see Table 1). In contrast, significantly fewer (12% of 160) NGF-treated cells microinjected with the Y13-259 antibody had neurites. Similarly, microinjection of the Y13-259 antibody significantly inhibited neurite outgrowth in response to bFGF. Of these cells, 25% of those microinjected with the control solution had neurites; whereas, 14% of those microinjected with the antibody had neurites (see Table 1). The data in Fig. 3(b) shows the percentages of neurite-bearing cells that were scored for each independent experiment. Microinjection of the antibody resulted in a greater number of points for which the percentage of neurite-bearing cells was lower than those in the control.

When bFGF was omitted for the 24 h postinjection, only 7–8% of the injected cells had neurites after microinjection of either control or antibody-containing solution. This percentage is significantly lower than the percentage of neurite-bearing cells following microinjection of the antibody in the presence of growth factor (12–14%). This difference indicates that only partial inhibition of the effects of the growth factors on neurite outgrowth was

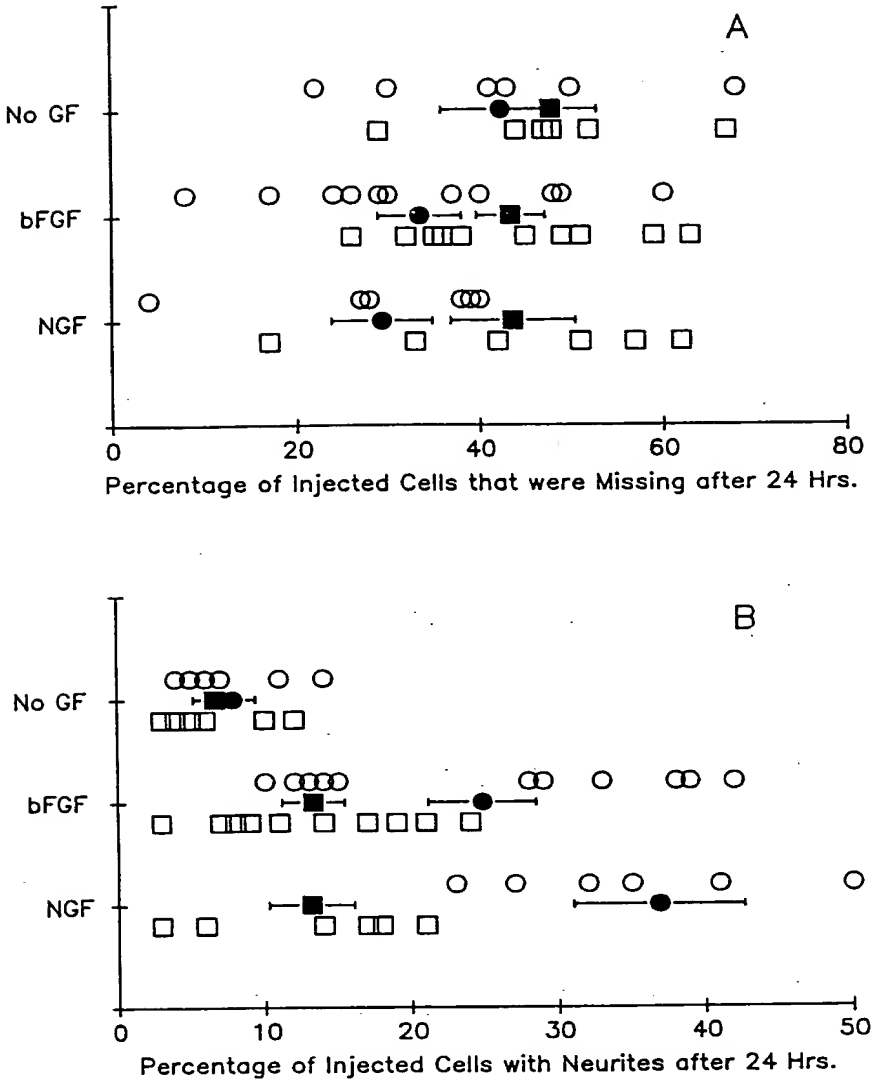

FIGURE 3. Distribution of the percentages of microinjected cells that were healthy or that had neurites. PC12 cells pretreated with either NGF or bFGF were microinjected with either control solution (circles) or the solution containing Y13-259 antibody (squares). Between 20–60 cells were microinjected at each 'site' (or experimental sitting); these microinjections were performed with the same micropipette. The mean number of cells injected per site was 30.0 ± 1.7 (SEM). Each open symbol corresponds to the percentage of microinjected cells that were scored as missing (A), or scored as having neurites (B), at a particular microinjection site. These points were offset vertically for clarity. The means and SEMs for each set of points is shown by the solid symbols. The condition 'No GF' corresponds to cells that were pretreated with bFGF, but no growth factor was present for the 24 h postinjection.

achieved under these conditions. This partial inhibition could be due to either incomplete blockade of p21*ras* function or to a second signaling pathway that does not involve p21*ras*. Evidence against the first possibility comes from the observation that the percentage of missing cells was equally increased by growth factor deprivation or by antibody microinjection (see Table 1). Thus, microinjection of the antibody was associated with a retraction of established neurites and/or inhibition of neurite outgrowth induced by these growth factors.

DISCUSSION

Microinjection of the antibody Y13-259 into NGF-treated and bFGF-treated PC12 cells causes a retraction of established neurites and an inhibition of further neurite outgrowth by these growth factors. Since Y13-259 binds p21*ras* and blocks its ability to function, the reduced percentage of neurite-bearing cells suggests that p21*ras* is involved in the maintenance of established neurites by both NGF and bFGF. Microinjection of the antibody into unprimed

cells suggest that initial induction of neurite outgrowth is also mediated by p21*ras* (data not shown). Since the induction of neurite outgrowth by bFGF, like that of NGF, requires p21*ras* function, the protein p21*ras* may be a common effector in the pathway by which these growth factors elicit neurite outgrowth in PC12 cells.

The microinjection of high concentrations of antibodies into single PC12 cells was thought to be difficult owing to the small size of the cells (10–20 μm diameter) and the relatively high viscosity of the antibody solution, which limits the size of the micropipette that can be used for successful impalement. In preliminary experiments, we found that good survival of microinjected cells could be achieved by attention to the following conditions: the pretreatment of the cells with growth factor (either NGF or bFGF), the size of the pipette tip, the volume of material injected, and the composition of the medium bathing the cells during and shortly after impalement. By refining our microinjection procedure, we improved the viability of native PC12 cells following impalement sufficiently to obtain statistically significant and reproducible results.

There is evidence that the levels of Kirsten c-*ras* and Harvey c-*ras* mRNA are increased following treatment of PC12 cells with NGF (Curran and Morgan, 1985). A role for p21*ras* in the induction of neurite outgrowth by neurotrophic factors is supported also by the observation that levels of expression of a v-Ha-*ras* gene transfected into PC12 cells correlates well with the expression of neuron-associated properties (Sugimoto et al., 1988). While the observed increase in the level of p21*ras* is not sufficient to demonstrate a role for this protein in the action of NGF, further evidence can be obtained by blocking its activity. The monoclonal antibody Y13-259 was generated against v-Ha-p21*ras* and recognizes all rat p21*ras* species (Furth et al., 1982). Although it remains possible that the antibody may have slight crossreactivity with other GTP-binding proteins (Beckner, Hattori and Shih, 1985), no high-affinity binding with these has yet been reported. In in vitro studies of how Y13-259 blocks activity, it has been found that the antibody does not directly interfere with GTP binding, the GTPase activity or the autophosphorylation activity of p21*ras* (Lacal and Aaronson, 1986), but acts by inhibiting GDP/GTP exchange (Hattori et al., 1987) and by blocking the interaction of p21*ras* with the GTPase-activating protein (GAP) (Srivastava, Di Donato and Lacal, 1989). Our results therefore support the evidence that p21*ras* is involved in the mechanism of action of NGF in native PC12 cells and, furthermore, complement similar findings obtained by microinjection of the Y13-259 antibody into fused PC12 cells (Hagag, Halegoua and Viola, 1986; Kremer, Brugge and Halegoua, 1989). In addition, our results provide evidence that bFGF-induced neurite outgrowth is at least partly mediated by p21*ras*.

Microinjection of the antibody Y13-259 into PC12 cells resulted in a statistically significant increase in the number of cells that were scored as missing (see Table 1). It is known that NGF and bFGF can promote the survival of neuronal cells in culture (Walicke et al., 1986; Morrison et al., 1986) and the survival of PC12 cells cultured under serum-free conditions (Greene and Tischler, 1982; Togari et al., 1985; Schubert, Ling and Baird, 1987; Rydel and Greene, 1987). Our experiments, in which cells microinjected with the control solution were either treated or not treated with growth factor, show that the absence of the growth factor increased the number of missing cells. While the true fate of these missing cells is not known, cell morbidity and mortality probably make major contributions to the number of missing cells. Another possibility is a reduction in cell adhesion to the collagen substrate. However it occurs it is clear that the mechanism behind cell loss can be affected by either the absence of growth factor or introduction of Y13-259, because either of these conditions results in a significantly greater number of missing cells than does microinjection of the control solution in the presence of a growth factor. Thus, these results demonstrate that NGF and bFGF can decrease the number of missing cells, and that this effect operates through a pathway that involves p21*ras*. Our results suggest, therefore, that as well as being involved in the signaling pathway leading to neurite outgrowth, p21*ras* is involved in the increase in cell number by NGF and bFGF under these conditions; both responses are positively regulated by this protein. These findings are consistent with the recent report that p21*ras* promotes survival and fiber outgrowth in cultured neurons (Borasio et al., 1989).

The mechanism by which NGF and bFGF activate p21*ras* is not known. The reported sequence of the bFGF receptor from chick embryos contains a tyrosine kinase in its cytoplasmic domain (Lee et al., 1989). The NGF receptor for PC12 cells lacks any obvious catalytic activity in its rather small cytoplasmic domain (Radeke et al., 1987), but there is evidence that a tyrosine kinase may associate with

this receptor following NGF binding (Maher, 1988, 1989). Moreover, introduction of the v-*src* gene encoding for the tyrosine kinase $pp60^{v\text{-}src}$ into either PC12 cells (Alema et al., 1985; Rausch et al., 1989), or the unresponsive variant cells PC12nnr5 (Eveleth et al., 1989), stimulates neurite outgrowth. Thus activation of a tyrosine kinase following the binding of NGF and bFGF to their respective receptors in PC12 cells could lead to an activation of the GTPase activity of p21*ras*, presumably through a mechanism involving GAP, as has been proposed for other systems (Vogel et al., 1988; Santos and Nebreda, 1989; Molloy et al., 1989).

The mechanism by which p21*ras* acts to induce neurite outgrowth is unclear. Some studies suggest a role for protein kinase C in neurite induction by NGF. NGF has been shown to activate protein kinase C in PC12 cells (Hama, Huang and Guroff, 1986; Hama et al., 1987; Hall et al., 1988), and both NGF and bFGF stimulate the production of 1,2-diacylglycerol (a physiological activator of protein kinase C; Nishizuka, 1986), probably from sources other than the phosphinositides (Altin and Bradshaw, 1990). However, downregulation of protein kinase C by prolonged treatment with phorbol esters does not block neurite outgrowth or expression of early response genes such as c-*fos* and TIS genes, suggesting that both NGF and bFGF may induce these responses independently of protein kinase C (Reinhold and Neet, 1989; Sigmund et al., 1990; Kujubu et al., 1987; Altin et al., 1990). Similarly, some effects of p21*ras* in NIH 3T3 cells (Maly et al., 1989) and Swiss 3T3 cells (Lloyd et al., 1989) also may occur through a protein kinase C-independent mechanism. Nonetheless, *ras*-transformed cells have elevated levels of 1,2-diacylglycerol (Lacal, Moscat and Aaronson, 1987; Huang et al., 1988; Kato, Kawai and Takenawa, 1988; Lacal, 1990) suggesting that p21*ras* may lead to an activation of protein kinase C. Increased levels of 1,2-diacylglycerol can occur through the action of phospholipases, and there is some evidence that p21*ras* may be involved in the regulation of phospholipase activity. For example, p21*ras* induces membrane ruffling and local disruption of the phospholipid bilayer (Bar-Sagi and Feramisco, 1986; Montgomery, Jagger and Bailey, 1988), and exists in spatial proximity with phospholipase A_2 in the ruffles of cells transformed by *ras* genes (Bar-Sagi et al., 1988). Further, a recent study suggests that *ras*-mediated induction of DNA synthesis in NIH 3T3 cells requires phospholipase C activity (Smith et al., 1990). It has been purported, however, that p21*ras* is not involved in regulating the actions of either phospholipases C or A_2 (Yu, Tsai and Stacey, 1988).

Some evidence suggests that another effector downstream of p21*ras* might be GAP itself (Cales et al., 1988; McCormick, 1989; Garrett et al., 1989). In PC12 cells there is evidence which suggests that differentiation induced by *ras* may occur through a mechanism which involves the proto-oncogenes *fos* and *jun* (Sassone-Corsi, Der and Verma, 1989; Wu et al., 1989). Our present study clearly shows that p21*ras* is involved in the stimulation of neurite outgrowth and promotion of cell survival by NGF and bFGF in PC12 cells, but clarification of the precise role of p21*ras* in this action awaits further study.

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Testing the In Vivo Role of Protein Kinase C and c-Fos in Neurite Outgrowth by Microinjection of Antibodies into PC12 Cells

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To define the molecular bases of growth factor-induced signal transduction pathways, antibodies known to block the activity of either protein kinase C (PKC) or the *fos* protein were introduced into PC12 cells by microinjection. The antibody against PKC significantly inhibited neurite outgrowth when scored 24 h after microinjection and exposure to nerve growth factor (NGF). Microinjection of antibodies to *fos* significantly increased the percentage of neurite-bearing cells after exposure to either NGF or basic fibroblast growth factor (bFGF) but inhibited the stimulation of DNA synthesis by serum, suggesting that in PC12 cells, *fos* is involved in cellular proliferation. Thus, activation of PKC is involved in the induction of neurite outgrowth by NGF, but expression of the *fos* protein, which is induced by both NGF and bFGF, is not necessary and inhibits neurite outgrowth.

INTRODUCTION

Despite considerable knowledge about several polypeptide growth factors and their receptors, the molecular signaling mechanism(s) involved in differentiation and proliferation remains to be clearly delineated. The rat pheochromocytoma cell line PC12 has been used widely to study neuronal differentiation (Greene and Tischler, 1982) and is a useful system in which to distinguish those intracellular signals that may be specific for inducing differentiation on the one hand from those that induce proliferation on the other. PC12 cells proliferate as round chromaffin-like cells when grown in standard culture conditions, but on addition of nerve growth factor (NGF) or basic fibroblast growth factor (bFGF), the cells stop dividing, extend numerous processes, and display characteristics of fully differentiated sympathetic neurons (Green and Tischler, 1982; Togari *et al.*, 1985; Rydel and Green, 1987; Schubert *et al.*, 1987). Neuronal differentiation of PC12 cells can be induced by the *ras* or *src* transforming proteins (Alema *et al.*, 1985; Bar-Sagi and Feramisco, 1985; Satoh *et al.*, 1987; Eveleth *et al.*, 1989; Rausch *et al.*, 1989). Microinjection of antibodies that block the function of either the *ras* or the *src* proteins inhibits the induction of neurite outgrowth by NGF or by bFGF in both fused (Hagag *et al.*, 1986) and native PC12 cells (Altin *et al.*, 1991b), suggesting that these proteins are essential components of signal transduction pathways leading to differentiation in response to NGF and bFGF in PC12 cells.

In certain cell types, some actions of the *ras* protein may involve protein kinase C (PKC) (Nishizuka, 1986; Lacal *et al.*, 1987; Huang *et al.*, 1988; Lacal, 1990). Consistent with this view, both NGF and bFGF increase the production of 1,2-diacylglycerol, a physiological activator of protein PKC, in PC12 cells (Altin and Bradshaw, 1990) and NGF increases PKC activity (Cremins *et al.*, 1986; Hama *et al.*, 1986; Heasley and Johnson, 1989). Evidence suggesting that activation of PKC is necessary for induction of neurite outgrowth by NGF comes from the findings that sphingosine, an inhibitor of PKC, inhibits neurite outgrowth in PC12 cells (Hall *et al.*, 1988). In addition, tetradecanoyl phorbol acetate (TPA, an activator of PKC) induces neurite outgrowth in chick sensory ganglia explants (Hsu *et al.*, 1989) and potentiates

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the action of NGF in PC12 cells (Burstein *et al.*, 1982). However, some reports indicate that treatment of PC12 cells with TPA alone does not induce neurite outgrowth (Burstein *et al.*, 1982; but see Glowacka and Wagner, 1990) and that down-regulation of PKC by prolonged treatment with TPA does not block neurite outgrowth by NGF (Reinhold and Neet, 1989). These latter observations suggest that activation of PKC may be neither necessary nor sufficient for induction of this response.

Another potential signal for differentiation of PC12 cells is the oncogene c-*fos*, which is rapidly and transiently induced in these cells by both NGF and bFGF (Curran and Morgan, 1985; Milbrandt, 1986; Sheng *et al.*, 1988) and belongs to a class of genes referred to as immediate early genes. These genes are induced rapidly in many cell types by a number of mitogenic and differentiative stimuli in the presence of inhibitors of protein synthesis (for a recent review, see Sheng and Greenberg, 1990). The c-*fos* gene encodes a highly phosphorylated protein of 380 amino acids that localizes in the nucleus where it interacts with members of the *jun* family of proteins. The *fos* and *jun* protein families make up a major part of the activator protein-1 (AP-1) transcriptional complex that plays a role in regulating the transcription of genes possessing AP-1 promoters (Chiu *et al.*, 1988; Sheng and Greenberg, 1990). Increased AP-1 DNA-binding activity has been found in nuclear extracts of *ras*-infected PC12 cells, suggesting a possible involvement of *fos* and *jun* in the action of *ras* (Sassone-Corsi *et al.*, 1989; Wu *et al.*, 1989). That *fos* is an effector downstream of *ras* is supported also by studies in other cell types, NIH 3T3 and rat 208F fibroblast cells, which show that microinjection of the transforming *ras* protein causes a rapid induction of c-*fos* (Stacey *et al.*, 1987; Riabowol *et al.*, 1988a) and that the expression of antisense-c-*fos* mRNA reverses the transforming effects of the *ras* oncogene (Ledwith *et al.*, 1990). Thus, induction of c-*fos* may constitute an essential part of the signal for differentiation by NGF and bFGF in PC12 cells. Conversely, the transfection of a mouse N-*ras* gene into a subline of PC12 cells was seen to induce neurite outgrowth without induction of the c-*fos* gene (Guerrero *et al.*, 1986, 1988), and overexpression of the c-*fos* gene in PC12 cells was reported to inhibit neurite induction by NGF (Ito *et al.*, 1989).

Recently, affinity-purified antibodies against the *fos* protein have been developed that inhibit the ability of serum to stimulate DNA synthesis in rat fibroblasts (Riabowol *et al.*, 1988b). To determine whether the *fos* protein plays a role in the neurotrophic action of NGF and bFGF, we have scored the ability of PC12 cells to respond morphologically to NGF and bFGF in the absence and presence of microinjected anti-*fos* antibodies. In addition, we have studied the effects of microinjecting a monoclonal antibody to PKC (monoclonal antibody PKC 1.9), which is reported to inhibit the activity of all PKC isozymes (Mochly-Rosen and Koshland, 1987, 1988). Our results indicate that microinjection of the PKC antibody inhibits neurite induction by NGF but that *fos* antibodies significantly potentiate the induction of neurite outgrowth in response to either NGF or bFGF.

MATERIALS AND METHODS

Materials

Monoclonal antibody PKC 1.9 was obtained from GIBCO-BRL (Grand Island, NY). The affinity-purified antibodies directed against the *fos* protein used in the present experiments were identical to those described previously (Riabowol *et al.*, 1988b). NGF was prepared as β-NGF by the method of Mobley *et al.* (1976). A bFGF analogue in which all half-cystine residues were replaced by serines (Fox *et al.*, 1988) was used in all experiments with bFGF. This analogue was kindly provided by Dr. Gary M. Fox, Amgen Inc. (Thousand Oaks, CA) and previously was found to be indistinguishable from preparations of the natural protein in stimulating neurite outgrowth and phospholipid metabolism in PC12 cells (Altin and Bradshaw, 1990).

Cell Culture and Microinjection

PC12 cells were adapted to grow in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 5% heat-inactivated horse serum, and 1% Fungi-Bact solution as described previously (Altin *et al.*, 1991b). PC12 cells were seeded in collagen-coated (Greene and Tischler, 1982) six-well plates (Linbro Flow Laboratories, McLean, VA) at a density of 4×10 cells/cm^2 in low serum media (DMEM supplemented with 2% fetal calf serum and 1% heat-inactivated horse serum) 48 h before microinjection. To buffer the medium while the cells were out of the incubator, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (20 mM, pH 7.4) was added to the medium after removal from the incubator. Immediately before microinjection, the medium was replaced with warmed Dulbecco's phosphate-buffered saline (pH 7.4), which also contained 10 mM glucose. After microinjection, the cells were kept in this solution for 1–2 h at 37°C before replacing with low serum media (as above) in the presence of either NGF (100 ng/ml) or bFGF (5 ng/ml).

Individual cells were microinjected with either a control solution or an antibody solution with the use of techniques similar to those used for microinjection of the anti-p21*ras* antibody (Altin *et al.*, 1991b). Each culture well contained both control-injected cells and antibody-injected cells; this controlled effectively for any variability in the amount of induction by the growth factor. The control solution was composed of 60 mM KCl, 10 mM NaCl, 0.1 mM EDTA, and 100 mg/ml rhodamine-conjugated dextran (70 kDa, #D-1818, Molecular Probes, Eugene, OR); the antibody solution was control solution plus either PKC 1.9 antibody (5 mg/ml) or affinity-purified antibodies to *fos* (8 mg/ml). In previous microinjection studies, it was shown that the use of this control solution gave results that were indistinguishable from those in which the control solution also contained 8 mg/ml of bovine serum albumin or an equivalent amount of antibody that was inactivated by freeze-thawing (Altin *et al.*, 1991b). Most cells received injections of ~10% of the cell volume, but due to the small volume of the PC12 cells and the consequent difficulty of their microinjection, the amount of solution expelled was variable. The total number of rhodamine-labeled cells was counted after microinjection of a group of cells, so that any cells (healthy or not) that detached immediately were not included in the count. At ~24 h postinjection, the rhodamine-labeled cells were examined and scored as described previously (Altin *et al.*, 1991b). This was the optimal time for scoring the cells because the levels of the injected antibodies declined after 24 h (see Altin *et al.*, 1991b) and because the intensity of the rhodamine fluorescence was notably decreased at 48 h compared with 24 h (Altin and Wetts, unpublished observations).

Table 1. The percentage of PC12 cells that were scored into one of four different categories 24 h after microinjection of either control solution or a solution containing the PKC 1.9 antibody

Condition	Cells (%)				Total cells injected	Number of experiments
	Neurites	Round	Abnormal	Missing		
NGF (control)	38	31	27	4	277	6
PKC-1.9 antibody[a]	27[b]	29	33[c]	11[b]	263	10

The four-by-two chi-square test for equality of two multinomials was used to compare all four categories simultaneously. There was a significant difference between the PKC-1.9 antibody-injected cells and control-injected cells treated with NGF ($^{a}p < 0.05$). On finding a difference with the chi-square test, the unequal pairs were identified by the Irwin-Fisher test, using the normal approximation to the hypergeometric distribution ($^{b}p < 0.01$; $^{c}p < 0.10$).

Statistical Analyses

Groups of 10–50 cells were microinjected with the same micropipette, and 4–10 groups were injected for each experimental condition (Tables 1 and 2). After 24 h, each injected cell was assigned to one of four categories (as described in RESULTS). To determine whether the differences between antibody- and control-injected cells were significant, statistical analyses, recommended by Dr. Howard Tucker (Department of Mathematics, University of California, Irvine), were performed. The number of cells in each category was summed across the groups, thereby providing equal importance to each injected cell regardless of the number of cells in a group. A four-by-two chi-square test was used first to detect any difference between antibody- and control-injected cells. This test simultaneously compares all four categories and indicates if there is any difference between the antibody- and control-injected cells. If there was an overall difference, the Irwin-Fisher test was used then to independently compare each of the four categories and to determine which one(s) contributed to the difference.

Immunocytochemistry Assay for DNA Synthesis

To identify the cells undergoing DNA synthesis after stimulation with serum, the cells were incubated in DMEM containing 12% fetal calf serum, 6% horse serum, and 1% Fungi-Bact solution in the presence of 10 μM bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO) during the 24 h after microinjection. The cells were rinsed with phosphate buffer (0.1 M, pH 7.4), fixed with 70% ethanol (30 min at room temperature), air-dried (5–10 min), and treated with 2 N HCl plus 0.5% Triton X-100 to permeabilize the cells and to denature the DNA (30 min). After washing with phosphate buffer, the cells were incubated with a solution of 20% goat serum plus 0.2% Triton X-100 in phosphate buffer, followed by incubation with anti-BrdU antibody (Becton Dickinson, San Jose, CA) diluted 1:20 in the same solution, rinsed three times in the same solution, and then incubated in fluorescein-conjugated goat anti-mouse IgG antibody diluted 1:100 in the same solution (30 min per incubation at room temperature). After three washes with phosphate buffer, rhodamine and fluorescein fluorescence was visualized to detect the presence of the dextran and the BrdU, respectively.

RESULTS

Microinjection of Antibodies into PC12 Cells

Microinjections were performed on PC12 cells that had been plated in low serum medium without growth factors for 48 h. Few of these cells had prominent neurites (<1%) and about one-half were spherical and poorly attached to the substrate with the remainder slightly

Table 2. The percentage of PC12 cells that were scored into one of four different categories 24 h after microinjection of either control solution or a solution containing Fos antibodies

Condition	Cells (%)				Total cells injected	Number of experiments
	Neurites	Round	Abnormal	Missing		
NGF (control)	32	29	26	13	195	6
Fos antibody[a]	39[b]	17[c]	23	20[c]	155	5
bFGF (control)	8	27	46	19	107	5
Fos antibody[a]	21[d]	18[c]	31[c]	30[c]	118	5
No GF (control)	4	52	31	13	126	5
Fos antibody[a]	2	38[d]	40[b]	20[c]	181	7
Hi serum (control)	0	47	12	41	115	4
Fos antibody	0	44	23	33	139	5

The four-by-two chi-square test for equality of two multinomials was used to compare all four categories simultaneously. There was a significant difference between the Fos antibody-injected cells and control-injected cells for the NGF-, bFGF-, and no growth factor (No GF)-treated cells ($^{a}p < 0.05$). On finding a difference with the chi-square test, the unequal pairs were identified by the Irwin-Fisher test, using the normal approximation to the hypergeometric distribution ($^{b}p < 0.10$; $^{c}p < 0.05$; $^{d}p < 0.01$). When the medium containing BrdU and high levels of serum was present after microinjection ("Hi serum" condition), there were no significant differences between control- and antibody-injected cells.

flattened and well attached. The latter cells were microinjected with either a control solution or an antibody solution, both of which contained equal concentrations of rhodamine dextran to permit identification of the injected cells (Wetts *et al.*, 1989; Altin *et al.*, 1991b). Rhodamine-labeled cells, examined 24 h after microinjection, were scored blind regarding the injected solution (either control or antibody-containing). Each clearly labeled cell was classified into one of three groups. Cells were scored as having *neurites* if they had processes of

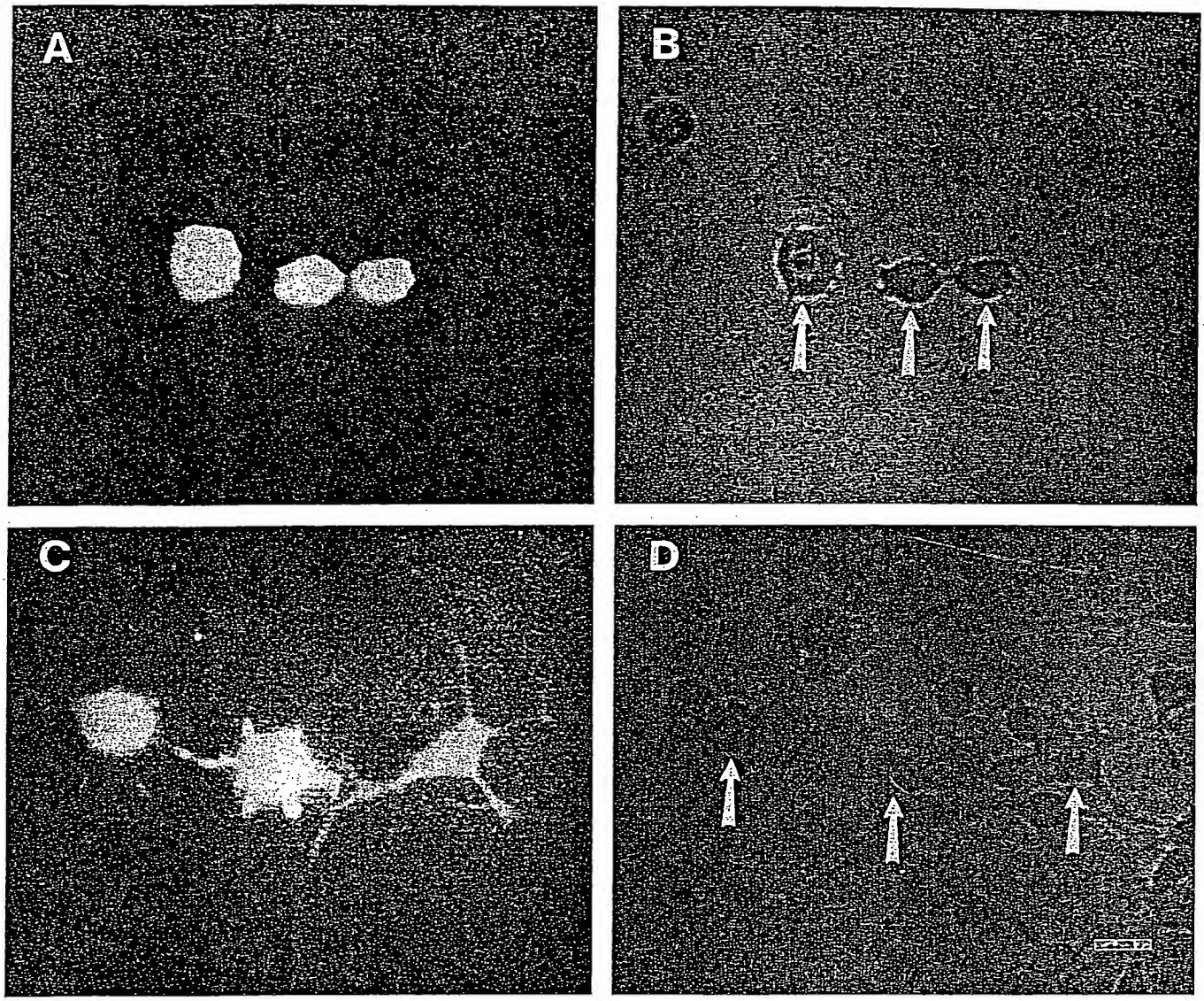


Figure 1. Effect of Fos antibodies on neurite induction by NGF. PC12 cells were plated for 48 h and then microinjected with either a control solution (A and B) or a solution containing antibodies to Fos (C and D). After exposure of the cells to NGF for 24 h, the microinjected cells were identified with rhodamine fluorescence (A and C). Bright fluorescence causes "blooming" in the image from the SIT video-camera, so the fluorescence images of these cells appear larger than their actual size. Treatment with NGF for 24 h is a relatively short period of time, so less than half of the uninjected cells had begun to extend neurites (45%). Of the cells injected with the control solution, a similar percentage responded to the NGF treatment (53%, from Table 1). This particular field (B) shows some of the cells that had not yet extended neurites (arrows); they appear indistinguishable from uninjected cells. A greater proportion of cells injected with the antibody solution had neurites (arrows) in comparison with control-injected cells (D). Similar results were seen when the cells were treated with bFGF instead of NGF (not shown). Scale bar, 20 μm.

at least one cell diameter in length or had multiple neurites. *Round* cells had small or no neurites. Round and neurite cells appeared to be normal and healthy. Other cells that may have been adversely affected by the microinjection procedure were scored as *abnormal*. These cells exhibited either very faint or punctate fluorescence. Under bright-field illumination, cells scored as abnormal were smaller, darker, and more granular than the normal cells. To determine the number of *missing* cells, the total number of labeled cells in these three groups was subtracted from the number of cells injected on the previous day. The cause(s) of this cell loss is unknown; it may reflect a decrease in cell attachment and/or cell survival.

Effect of Monoclonal Antibody PKC-1.9 on Neurite Outgrowth

To determine whether activation of PKC plays a role in the induction of neurite outgrowth by NGF, we microinjected antibodies that inhibit PKC activity (monoclonal antibody PKC 1.9) into native PC12 cells. Twenty-four hours after microinjection and continuous exposure to NGF, the percentage of neurite-bearing cells after antibody treatment (27%) was significantly lower than the percentage of cells that had neurites after microinjection of the control solution (38%) (see Table 1). However, injection of the antibody did not inhibit neurite outgrowth in all cells, because this percentage (27%) is greater than the percentage of neurite-bearing cells in the absence of NGF (2–4%) (Table 2). This incomplete inhibition could be due to the variability in the amount of antibody solution injected into each cell; that is, in some cells the amount of antibody was insufficient to block 100% of the PKC activity for the full 24 h. Alternatively, it is possible that a subpopulation of microinjected cells (perhaps those that were arrested or that were residing in a similar phase of the cell cycle) was more sensitive to PKC inhibition. Yet another possibility is that neurite outgrowth is partly mediated through PKC-independent pathways. Although it is incomplete, the observed inhibition suggests that PKC plays some role in the induction of neurite outgrowth by NGF.

Effect of PKC 1.9 Antibody on Cell Number

Microinjection of the PKC 1.9 antibody resulted in a significantly greater percentage of missing cells when scored 24 h after microinjection and exposure to NGF (Table 1). Thus, 4% of the cells that were microinjected with the control solution were missing as compared with 11% of those microinjected with the PKC 1.9 antibody ($p < 0.01$). The cause(s) of this cell loss is unknown; in the control group it could be due to the trauma of the microinjection procedure or to normal events of cell culture. However, the significant increase in the number of missing cells after antibody microinjection indicates that the antibody causes additional cell loss, perhaps by augmenting the factors involved in normal cell loss. Regardless of the mechanism, the increased cell loss is a specific effect of the PKC 1.9 antibody, because the effect was not observed after microinjection of other antibody solutions (e.g., Altin *et al.*, 1991b).

Effect of the fos Antibodies on Neurite Outgrowth

To determine whether the activity of the *fos* protein is necessary for the induction of neurite outgrowth, affin-

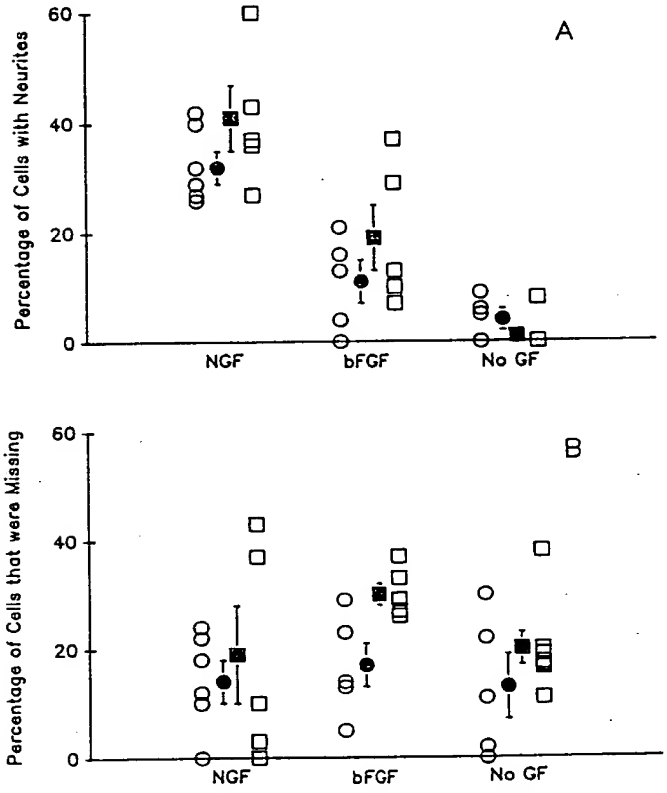
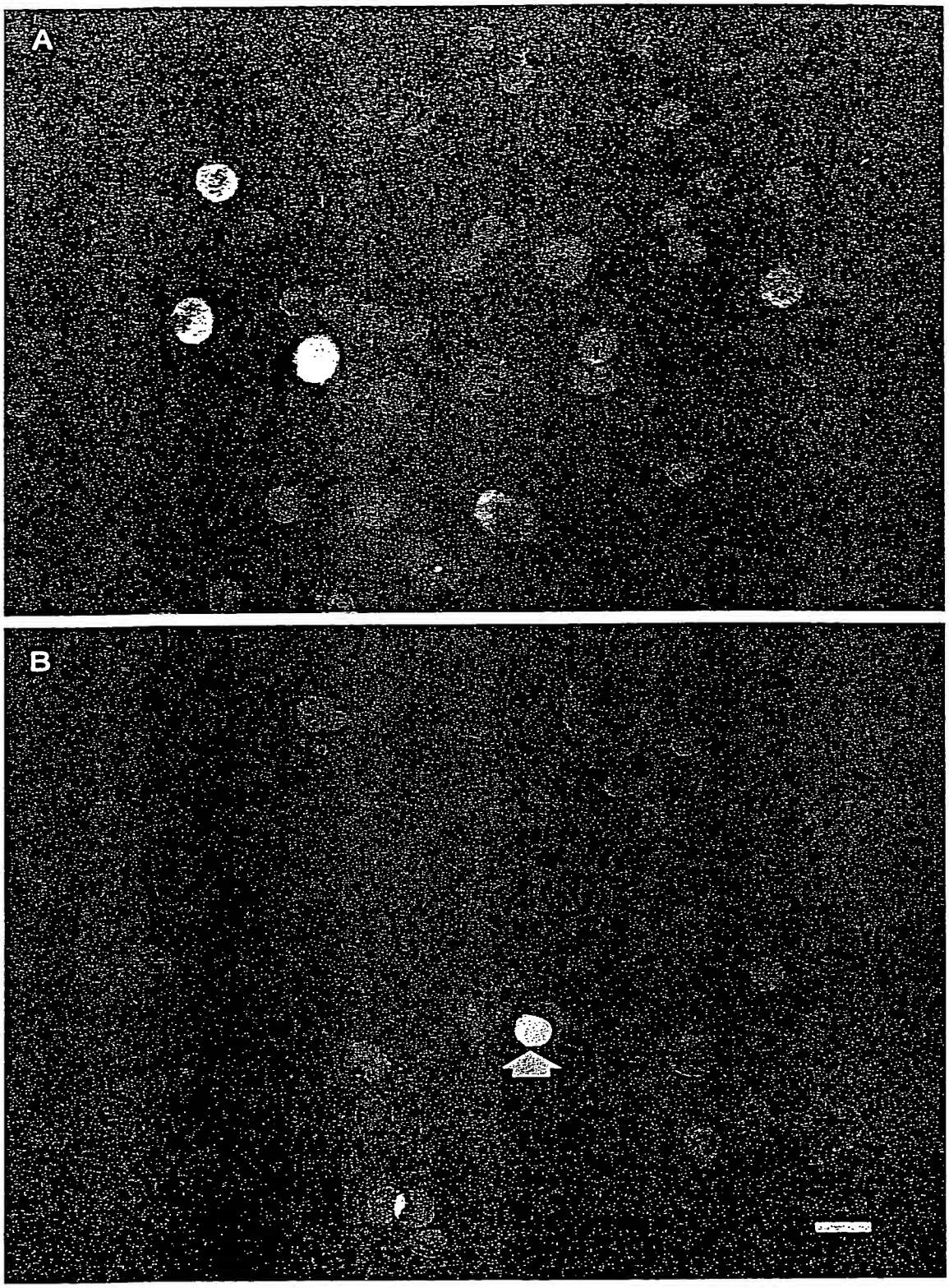


Figure 2. The percentages of microinjected PC12 cells that had neurites or that were missing 24 h after microinjection with either the control solution (circles, left) or the solution containing the Fos antibodies (squares, right). Between 10 and 50 cells were microinjected at each "site" (or experimental sitting); these microinjections were performed with the same micropipette. The mean number of cells injected per site was 26.7 ± 1.7 (SEM). Each open symbol corresponds to the percentage of microinjected cells that were scored as neurite-bearing (A) or scored as missing (B) at a particular microinjection site. The means and SEM for each set of points are shown by the open bars. The condition "No GF" corresponds to cells that had no growth factor present for the 24-h postinjection. The percentages of neurite-bearing cells (A) were different for the three conditions, indicating that NGF was more effective than bFGF (which was more effective than no GF) in inducing neurite outgrowth after 24 h. For both NGF and bFGF, microinjection of Fos antibodies resulted in an increased percentage of neurite-bearing cells in more experiments compared with control microinjections.


A
B

ity-purified antibodies directed against *fos* were microinjected into PC12 cells and the cells were scored for their ability to extend neurites in response to NGF or bFGF. In contrast to injection of the PKC-1.9 antibody, microinjection of the *fos* antibodies was associated with a significant increase in the number of cells with neurites induced by NGF (Figure 1, A and B and Figure 2A) and by bFGF (Figure 2A), relative to microinjection of the control solution (Figure 1, C and D and Figure 2A). Quantitatively, 32% of the control cells had neurites, whereas 39% of the cells microinjected with the *fos* antibodies had neurites after exposure to NGF (Table 2). Similarly, after exposure to bFGF, the percentage of cells with neurites was lower for control-injected cells (8%) than for antibody-injected cells (21%). This effect was a function of growth factor stimulation because the percentage of cells with neurites was essentially the same after control (4%) or antibody (2%) injection when growth factor was omitted during the 24 h postinjection (in addition to the 48 h preinjection) (Table 2 and Figure 2A). This suggests that the *fos* antibodies do not induce neurite outgrowth by themselves. However, in the presence of either NGF or bFGF, introduction of the antibodies appears to potentiate the action of the growth factors. These results suggest that the *fos* protein does not play a necessary role in neurite induction and in fact appears to inhibit this process.

Effect of fos Antibodies on Numbers of Round Cells and Cell Loss

In comparison with the controls, microinjection of *fos* antibodies resulted in a significant decrease in the proportion of round cells but in an increase in the percentage of missing cells 24 h after microinjection. Thus, for NGF- or bFGF-treated cells, the proportion of round cells decreased from 27 to 29% in cells microinjected with control solution to only 17–18% in cells microinjected with the antibodies (Table 2). The percentage of missing cells for NGF-treated cells increased from 13% in control-injected cells to 20% in antibody-injected cells, whereas for bFGF-treated cells the percentage increased from 19% in control-injected cells to 30% in antibody-injected cells (Table 2 and Figure 2B). In cells not treated with growth factor during the 24-h postinjection period, the percentage of round cells decreased from 52% in control-injected cells to 38% in antibody-injected cells, but the percentage of missing cells increased from 13% in control-injected cells to 20% in antibody-injected cells (Table 2 and Figure 2B). Because microinjection of the *fos* antibodies affects the proportion of round and missing cells in the absence, as well as the presence, of growth factor, the results suggest that the *fos* antibodies interfere with the basal level of *fos* activity in these cells (which were not treated with growth factor before microinjection) and that treatment with NGF or bFGF does not overcome the neutralizing effect achieved after introduction of the antibodies. This suggests that under these conditions *fos* activity (whether basal activity or induced by treatment with NGF or bFGF) plays at least some role in promoting cell attachment and/or cell survival.

Effect of fos Antibodies on DNA Synthesis

Because our studies suggest that c-*fos* expression is not required for neurite outgrowth, we tested the possibility that the *fos* protein is involved in cell growth. For these experiments, cells that had been plated for 48 h under low serum conditions (i.e., the same conditions as for the neurite outgrowth experiments) were microinjected with either the control solution or the solution containing *fos* antibodies and then were stimulated to divide with high-serum-containing medium in the presence of 10 μM BrdU for 24 h. The percentages of neurite-bearing, round, abnormal, or missing cells between control-injected and antibody-injected cells were not statistically significant (Table 2). After scoring morphology, these cells were processed for immunocytochemistry with fluorescein-labeled secondary antibodies against the BrdU-specific antibody. Fluorescein fluorescence shows the nuclei of cells that incorporated BrdU into their

Figure 3. Effect of Fos antibodies on the stimulation of DNA synthesis by serum. PC12 cells (plated for 48 h under low serum conditions) were microinjected with either a control solution (A) or a Fos antibody-containing solution (B) and then exposed to high serum medium containing the thymidine analogue BrdU. After 24 h, many of the uninjected cells were labeled by fluorescein fluorescence (green label), indicating that they had incorporated BrdU into their DNA. The microinjected cells were identified by rhodamine fluorescence (red label). Some cells microinjected with the control solution (A) had incorporated BrdU (the green and red labels together appear yellow), indicating that microinjection of cells does not inhibit passage through the cell cycle, although some cells had not begun to incorporate BrdU (red or orange label). Cells microinjected with the Fos antibodies (B) were unlabeled by fluorescein (only the red label is visible), indicating that the antibody had inhibited entry into S phase in these cells. One of the injected cells is on top of a fluorescein-labeled cell, hence it appears yellow but had not incorporated BrdU (arrow in B). A small proportion of cells injected with the antibodies were labeled by fluorescein, and this proportion is significantly smaller than the proportion of control-injected cells that were fluorescein-labeled (see Table 3). The fluorescein fluorescence of uninjected cells incubated in the presence of thymidine instead of BrdU is very low (not shown); this lack of labeling verifies the specificity of the anti-BrdU antibodies. A narrow-band emission filter was used for the fluorescein fluorescence pictures to eliminate any fluorescence from the rhodamine of the injected cells. To enhance the clarity of the video images, the original black-and-white images were pseudo-colored and combined (Vidim program courtesy of Dr. Scott Fraser, UCI). Scale bar, 20 μm.

DNA, indicating that these cells had entered into S phase during the labeling period. Most of the uninjected cells, as well as the control-injected cells (identified by rhodamine fluorescence), incorporated BrdU (Figure 3A). However, few antibody-injected cells (also rhodamine labeled) were labeled for BrdU (Figure 3B). The proportion of cells that displayed fluorescein fluorescence is significantly lower for cells microinjected with the antibody solution (30%) than for cells microinjected with the control solution (59%, Table 3), suggesting that the *fos* antibodies are effective in inhibiting the stimulation of DNA synthesis by serum in PC12 cells.

DISCUSSION

To study signal transduction pathways, we microinjected function-blocking antibodies into unfused native PC12 cells. Comparisons of antibody-injected cells with neighboring control-injected cells revealed small but significant differences in the percentage of cells bearing neurites after 24 h (Tables 1 and 2). There are several reasons why the observed differences were not larger. Some cells might not have gotten enough antibody to completely block the activity of PKC (or *fos*). The small size of native PC12 cells increased the technical difficulties of the microinjection technique, but artificially increasing the cell size by fusing together several cells might have caused changes in the normal transduction pathways. In addition, there is substantial evidence that signal transduction can involve multiple pathways, so that completely blocking one pathway may only cause a partial effect. Although we observed relatively small differences in the percentages of cells in each category, we microinjected many cells, and the statistical tests indicated that the observed differences were significant. Thus, our results provide evidence that PKC contributes to neurite outgrowth induced by NGF and that *fos* inhibits neurite outgrowth and is involved in proliferation.

Table 3. The percentage of dividing PC12 cells after microinjection of either control solution or Fos antibody solution.

Condition	Anti-BrdU-labeled cells (%)
Control	59
Fos antibody	30*
Uninjected	63

Cells were incubated with high-serum-containing medium and bromodeoxyuridine (BrdU) for 24 h before conducting immunocytochemistry to detect the incorporation of BrdU into DNA. Percentages for antibody-injected cells were significantly different from control-injected cells by chi-square test (*$p < 0.005$). The percentages for control-injected cells and uninjected cells were similar, indicating that the microinjection procedure does not inhibit cell division.

The monoclonal antibody anti-PKC 1.9, raised against highly purified rat brain PKC, recognizes an epitope in the catalytic domain of the enzyme (Mochly-Rosen and Koshland, 1987). The antibody has high specificity for PKC and strongly inhibits the activity of all PKC isozymes in vitro and in vivo without affecting either the cAMP-dependent protein kinase or the calcium/calmodulin-dependent kinase (Mochly-Rosen and Koshland, 1987, 1988). Our findings that microinjection of this antibody into PC12 cells inhibits NGF-induced neurite production provides independent evidence for a role for PKC in this process. This is consistent with the observations that TPA potentiates the action of NGF in PC12 cells (Burstein *et al.*, 1982) but appears inconsistent with the reports that NGF (and bFGF) can elicit neurite outgrowth in PC12 cells in which PKC has been down-regulated by prolonged treatment with TPA (Reinhold and Neet, 1989; Damon *et al.*, 1990; Sigmund *et al.*, 1990). However, down-regulating PKC in this manner may not be a totally effective means of eliminating all PKC isozymes (Cooper *et al.*, 1989). Furthermore, this type of experiment does not exclude phosphorylation events (by activation of PKC by TPA) before the down-regulation. The partial inhibition of immediate early gene responses to NGF in such cells (Sigmund *et al.*, 1990; Altin *et al.*, 1991a) favors the view that, in PC12 cells, blocking PKC affects NGF-induced neurite outgrowth.

A major finding from the present work is that microinjection of antibodies directed against the *fos* protein into PC12 cells significantly potentiates the induction of neurite outgrowth in response to NGF and bFGF. Previous studies have shown that NGF and bFGF rapidly induce c-*fos* mRNA expression in PC12 cells and therefore suggested a role for the *fos* protein in the induction of differentiation by these growth factors (Curran and Morgan, 1985; Milbrandt, 1986). The *fos* antibodies used in the present study have been found to immunoprecipitate c-*fos* and possibly the *fos*-related antigen, *fra*-1 (Riabowol *et al.*, 1988b; Vosatka *et al.*, 1989; Riabowol, unpublished observations). Because *jun* is precipitated also under nondenaturing conditions by virtue of its tight association with *fos*, it is likely that other proteins in the AP-1 complex are inactivated by these antibodies in vivo. In vitro studies have shown that these antibodies block 90–95% of specific AP-1 gel shift activity (Riabowol, Schiff, and Gilman, unpublished data). Our results therefore suggest that in PC12 cells, *fos* and other proteins, such as *fra*-1 and *jun*, are not required for the induction of neurite outgrowth by NGF or bFGF. Instead, the results suggest that the activity of *fos* (and/or an antigenically related proteins) results in an apparent inhibition of the neurotrophic response induced by these growth factors. This result

is consistent with recent observations that over-expression of the c-*fos* gene in PC12 cells blocks neurite outgrowth by NGF (Ito *et al.*, 1989) and that induced expression of the N-*ras* oncogene in a subline of PC12 cells can elicit neurite outgrowth in the absence of c-*fos* induction (Guerrero *et al.*, 1988).

The role of *fos* in the action of NGF and bFGF is still unclear. NGF-induced neurite induction in "unprimed" PC12 cells (unlike neurite regeneration in "primed" cells) is thought to be a transcription-dependent event (Burstein and Greene, 1978; but see Nichols *et al.*, 1989). It is not yet clear whether protein synthesis is required. There is evidence that a transcription-dependent increase in the rates of synthesis of several cytoplasmic and nuclear proteins occurs within the first few hours of NGF treatment (Tiercy and Shooter, 1986), and it has been suggested that ongoing translation is needed for the neurite response (Greene and Shooter, 1980). Increased transcription of c-*fos* mRNA and expression of *fos* protein by NGF and bFGF in PC12 cells are transient responses, with both returning to near-basal levels after 4 h (Kruijer *et al.*, 1985). c-*fos* induction may still play a role in mediating responses other than initial neurite outgrowth, as suggested by our observation that the *fos* antibodies inhibit cell attachment and/or cell survival and also by the recent report that c-*fos* is required for the induction of tyrosine hydroxylase gene expression by NGF (Gizang-Ginsberg and Ziff, 1990).

Of related significance is our observation that the microinjection of the *fos* antibodies into PC12 cells significantly inhibited the stimulation of DNA synthesis by serum (Table 3). These results are consistent with similar findings in rat fibroblasts using these same antibodies and support the proposed role of c-*fos* in traversing the G1 phase of the cell cycle and entry into DNA synthesis (Riabowol *et al.*, 1988b). This suggests that in PC12 cells *fos* is not required for induction of neurite outgrowth but instead is involved in a pathway in which the major effect culminates in cellular proliferation. Indeed, one initial effect of NGF and bFGF on PC12 cells is to stimulate a cycle of cell division (Greene and Tischler, 1982), and it is possible that the rapid induction of c-*fos* expression by these growth factors constitutes a step in that division. Recent work suggests that expression of the N-*ras* gene in a subline of PC12 cells inhibits the induction of c-*fos* by NGF and bFGF (Thomson *et al.*, 1990). Because the pathway of neurite induction by NGF and by bFGF is thought to involve *ras* (Hagag *et al.*, 1986; Altin *et al.*, 1991b), an action of these growth factors may be to block or suppress c-*fos* expression at some time after its initial induction. This action, as well as the elimination of any constitutively expressed c-*fos* by autoregulatory mechanisms, may be necessary for priming in these cells, which occurs in the early phase of the NGF and bFGF response before the stimulation of any neurite outgrowth (Greene and Tischler, 1982; Rydel and Greene, 1987).

The mechanisms by which NGF, bFGF, and other extracellular stimuli elicit increased transcription of c-*fos* or other immediate early response genes are not well understood but clearly involve both PKC-dependent and -independent pathways (Cho *et al.*, 1989; Sigmund *et al.*, 1990; Damon *et al.*, 1990; Altin *et al.*, 1991a: Graham and Gilman, 1991). Although tyrosine phosphorylation may be involved in these pathways, some early response genes can be induced by cAMP and by agents that elevate intracellular Ca^{2+} (Milbrandt, 1986; Morgan and Curran, 1986; Sheng *et al.*, 1990). Furthermore, neither the activation of PKC nor the induction of c-*fos* transcription are specific for differentiation in PC12 cells because they are induced by agents, such as epidermal growth factor, that do not lead to neurite outgrowth. The results presented here support the view that *fos* expression in PC12 cells is involved primarily in mitotic responses (that may be subsequently repressed by NGF and bFGF), whereas PKC, at least in the NGF response, plays a positive role in initial neurite extension. That PKC may not be obligatory illustrates the multifaceted and perhaps redundant responses that these factors can induce in this cell line.

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Cytotoxicity of photosensitizers camphorquinone and 9-fluorenone with visible light irradiation on a human submandibular-duct cell line *in vitro*

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Abstract

The cytotoxic effect of two types of photosensitizers (camphorquinone, CQ, a widely used aliphatic type and 9-fluorenone, 9F, an aromatic type) in the presence of 2-dimethylaminoethyl methacrylate (DM) as a reducing agent with exposure to visible light (350–550 nm) was examined in a human cell line. Cytotoxicity was evaluated in terms of the percentage of cell survival, and the production of reactive oxygen in living single cells was measured with an adherent cell analysis and sorting laser cytometer and a peroxide indicator. The amount of reactive oxygen generated in the cells irradiated in the 9F (1 mM, 3 min) system was about 9-fold greater than under the same conditions in the CQ system. Similarly, the decrease in cell survival in the 9F system was about 10-fold greater than in the CQ. Both the production of reactive oxygen in the cells and the decrease in cell survival paralleled the concentration of photosensitizers and the irradiation time. Although the cell-damaging effects with the CQ system were mild, at a higher dose (10 mM) and longer irradiation time (24 min) it produced cell survival equal to that in the 9F (1 mM, 3 min) system. These results suggest that in the case of irradiated photosensitizer systems, 9F was much more damaging to the cells than CQ, which damage probably occurred via free radicals involving reactive oxygen generation.

Keywords: Photosensitizer; Camphorquinone; 9-Fluorenone; Cytotoxicity; Reactive oxygen; Phototoxicity

1. Introduction

Resin systems are widely used in modern dentistry as restorative materials, bonding agents, cements, and prosthetic and orthopaedic devices (Lloyd and Scrimgeour, 1996). Although their physical properties are constantly being improved, the application of these systems is occasionally associated with irritation of the dental pulp (Stanley et al., 1967, 1975; Ogle et al., 1986), periodontium (Nasjleti et al., 1983), and with intraoral reactions (Baker et al., 1988; Hensten-Pettersen A. and Wictorin L. 1981).

The visible-light polymerizing resin systems are initiated by visible-light irradiation of a photosensitizer in the presence of a reducing agent, which generates many free radicals leading to the polymerization of monomers. There are numerous studies on the toxicity of resin components such as dimethacrylate monomers (Baker et al., 1988; Hanks et al., 1991). For example, Hanks et al. tested the toxicity of 11 components in terms of the degree of DNA synthesis, total protein content, and protein synthesis of cultured mammalian fibroblasts, and found that ethoxylated bis-phenol A dimethacrylate had the most toxic effects and that CQ

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Abbreviations: ACAS, adherent cell analysis and sorting; CQ, camphorquinone; DM, 2-dimethyl-aminoethyl methacrylate; 9F, 9-fluorenone; HBS, Hepes-buffered saline.

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and DM as photopolymerization initiators had low cytotoxicity compared with the other components. from their irreversibility.

As the leaching of components from composite filling materials has a potential impact on both the structural stability and the biocompatibility of materials. the elution of leachable components was studied by Ferracane (1994). who found that dimethacrylate and initiators were extractable. Although there is no way. and no reports so far on the subject. to determine clinically the amounts or traces of CQ released from resin materials. CQ is reportedly extractable from various composite materials *in vitro* (Spahl et al., 1991).

Illumination of a photosensitizer. particularly in the presence of reducing agents, generates many free radicals (Allen, 1982). resulting in the initation and propagation of the polymerization of dimethacrylate monomers. However. damaging effects of free radicals on living cells. caused by the photoinitiator system. have been scarcely reported. Free radicals generated from resin systems might produce reactive oxygen in the biological system with oxygen because oxygen as a diradical reacts with polymerization radicals. The biocompatibility of CQ has not been sufficiently clarified. particularly in photosensitization. Thus we undertook this investigation to establish whether phototoxicity. measured in terms of survival of cultured cells. i.e. human submandibular-gland cells. occurs in the CQ and 9F systems following visible-light irradiation. Furthermore. the production of reactive oxygen induced by irradiation in the above systems in the living cells was determined by ACAS laser cytometer and a fluorescent indicator of peroxide.

2. Materials and methods

2.1. Reagents

CQ. 9F. and DM were obtained from Tokyo Kasei Chem. Co. (Tokyo). 5-(and -6)-carboxy-2',7'-dichlorofluorescin diacetate was purchased from Molecular Probes. Inc. (Eugene. OR). Superoxide dismutase. catalase. and Eagle's minimum essential medium were obtained from Sigma Chemical Co. (St. Louis. MO): newborn calf serum was obtained from JRH Biosciences.

2.2. Culture of cells

The cell line was a gift from Professor Dr M. Sato (Tokushima University) (Shirasuna et al., 1981) and has been maintained in our laboratory for 3 years. These cells were grown in Eagles' minimum essential medium supplemented with 10% newborn calf serum: cells were dispersed from the culture dishes with 0.01% trypsin/0.004 M EDTA and passaged once a week at a 1:10 dilution. This cell line has been used to study the mechanism of signal transduction in our laboratory using a laser cytometric method (Atsumi et al.. 1996). We used the human submandibular-duct cells for this study because of their characteristic tendency to form both a uniform monolayer and relatively isolated colonies and to give reproducible results due to keeping an equal intensity of illumination on the cells.

2.3. Measurement of reactive oxygen

A dichlorofluorescein assay for the quantification of intracellular reactive oxygen was used (Boissy et al.. 1989). In brief. 5-(and -6)-carboxy-2',7'-dichlorofluorescin diacetate is a non-fluorescent and stable ester compound that readily diffuses through cell membranes. Intracellularly. cytosolic esterase enzymes cleave the acetate moiety. and the resultant hydrolysed compound is trapped within the cell. In the presence of peroxides or hydroperoxide. the compound is oxidized to the highly fluorescent form of 5-(and -6)-carboxy-2',7'-dichlorofluorescein. As all reactive oxygen species form peroxide in living cells. these species were detected by measurement of the fluorescenee intensity of 5-(and -6)-carboxy-2',7'-dichlorofluorescein.

Preparation of cells was modified from our previous report (Atsumi et al., 1996) as follows. Submandibular-duct cells were inoculated in 35-mm glass-bottomed dishes at a density of 3×10^4 cells in 1 ml of Eagles' minimum essential medium supplemented with 10% newborn calf serum, and cultured at 37°C under 5% CO_2 for 2 days. The cells were then washed twice, and the medium was exchanged for one containing 1% serum 1 hr before the assay. This 1% serum was added to prevent cell detachment on addition of the fluorescent dye: it gave no statistically significant variation from serum-free medium in terms of cell survival or production of reactive oxygen. Both 10 μM 5-(and -6)-carboxy-2',7'-dichlorofluorescin diacetate, i.e. the indicator of reactive oxygen. and 0 1 mM 9F or CQ and DM at the same molar concentration were added to the cells in Eagles' minimum essential medium containing 1% newborn calf serum and incubated for 30 min. The excess dye and photosensitizer were removed by rinsing the dishes twice, and 1 ml of fresh HBS (145 mM NaCl. 5 mM KCl. 1 mM $CaCl_2$, 0.5 mM $MgSO_4$. 5 mM glucose. 10 mM Hepes. pH 7.4) was added. followed by irradiation at room temperature with visible light (350 550 nm, $\lambda_{max} = 500$ nm) for 0 3 min from a pencil-type dental lamp (Translux: Kulzer Co., Germany). The distance for exposure of the cells was fixed at 1 cm from the lamp.

The cells were then analysed immediately with an ACAS 570 laser-interactive cytometer (Meridian Co.,

Tokyo), a computer-controlled, scanning laser instrument operated by an image-analysis program. The instrument was equipped with an argon laser set at 488 nm, and the emission at 530 ± 30 nm was recorded as a pseudocolour from two-dimensional image scans by a 0.1-μm laser beam. Fluorescence intensity of approx. 100 single cells was gathered from 8–12 stages to make a histogram. Some scavengers of reactive oxygen species, such as histidine, superoxide dismutase, dimethyl sulphoxide, and catalase, were added separately to the cells at 1 min before irradiation.

2.4. Cytotoxicity testing

Submandibular cells were inoculated into 24-well plates (wells 14-mm dia) at a density of 3×10^4 cells/well in 1 ml of Eagles' minimum essential medium supplemented with 10% newborn calf serum and cultured at 37°C for 2 days under 5% CO_2. The medium was exchanged for serum-free medium 1 hr before the assay. Photosensitizer CQ or 9F and the same molar concentration of DM as reducing agent dissolved in ethanol solution were added to the cells, giving a final ethanol concentration of 1% for each test substance, and cells were then incubated for 30 min at 37°C. To remove excess reagent, the cells were washed twice with HBS. Fresh HBS was then added, the cells were irradiated with visible light (350–550 nm) for 0–5 min from the dental lamp (Kulzer) and then incubated in the dark at 37°C under 5% CO_2 for 4 hr. At the end of the incubation, the cells were dispersed from the dishes with 0.01% trypsin/0.004 M EDTA, and the number of living cells from each well was counted with a Burker–Turk haemacytometer.

The cytotoxicological end-point was evaluated by both cell detachment from the dish and staining with trypan blue (0.04%) to distinguish dead from living cells. Cytotoxicity was calculated from the number of living cells from experimental wells and from control wells (no photosensitizer and/or no irradiation). The results were statistically analysed by Student's *t*-test.

3. Results

3.1. Reactive oxygen generated in cultured cells by irradiated photosensitizers

Figure 1 shows the two-dimensional image scan data as pseudocolours for reactive oxygen generated in single cells following the addition of photosensitizer (1 mM 9F or 1 mM CQ) and 1 mM DM with visible-light irradiation for 3 min. The control cell (neither photosensitizer nor light) showed little fluorescence intensity indicating reactive oxygen. Addition of the photosensitizer and irradiation with visible light produced a great deal of reactive oxygen in the cell, and the amount induced from the 9F system was obviously greater than from the CQ system. The intensity in the central area of the cell, thought to be nuclear, was relatively weak, whereas that more peripheral, thought to be cytoplasmic, was strong irrespective of the use of 9F or CQ.

Next, image-scan data from about 100 single cells (like those in Fig. 1) from each system (9F and CQ) and the control were gathered, and line histograms were made for the fluorescence intensity of reactive oxygen in the cells (Fig. 2). The average value of intensity in the control was 150 ± 10, that of the CQ system was 270 ± 50, and that of the 9F system 2500 ± 500. Accordingly, the amount of reactive oxygen generated from the CQ system was about 2-fold greater than from the control but was only 1/9 that of the 9F system.

Figure 3 demonstrates that the production of reactive oxygen by the 9F system in the cells increased as irradiation time increased. The average values of intensity were as follows: 180 sec, 2500 ± 500; 90 sec, 2000 ± 400; 30 sec, 1000 ± 300; 0 sec, 200 ± 30. It is thus clear that reactive oxygen induced in the cell by irradiation in the 9F system was dependent on irradiation time.

The production of reactive oxygen increased as the concentration of 9F increased (Fig. 4). The average values of intensity were as follows: 1 mM, 2500 ± 500; 0.5 mM, 500 ± 250; control (0 mM), 150 ± 10. The dose-dependency is clearly evident.

Table 1 shows the radical-scavenging effects of some scavengers of reactive oxygen species on the 9F system. Histidine is a powerful quenching agent for singlet oxygen (Kukreja et al., 1993). Addition of histidine (25 mM), dimethyl sulphoxide (150 mM), superoxide dismutase (300 U/ml), or catalase (300 U/ml) reduced the generation of reactive oxygen in the 9F (1 mM-3 min) system. Consequently, the 9F system appears to generate not only singlet oxygen but also reactive oxygen species such as hydroxy radical, superoxide anion, and hydrogen peroxide.

3.2. Cell damage caused by irradiated photosensitizers

Damage to the cells caused by irradiation of 9F or CQ in the presence of DM was investigated in terms of their survival (Fig. 5). When the photosensitizer was not added to the cells (control), survival was unchanged with or without visible-light irradiation for 3 min. By the addition of 1 mM 9F, the cell survival was 78% without irradiation, and it dropped to 39% with irradiation for 3 min; furthermore, it significantly recovered to 72% by the addition of hydroquinone

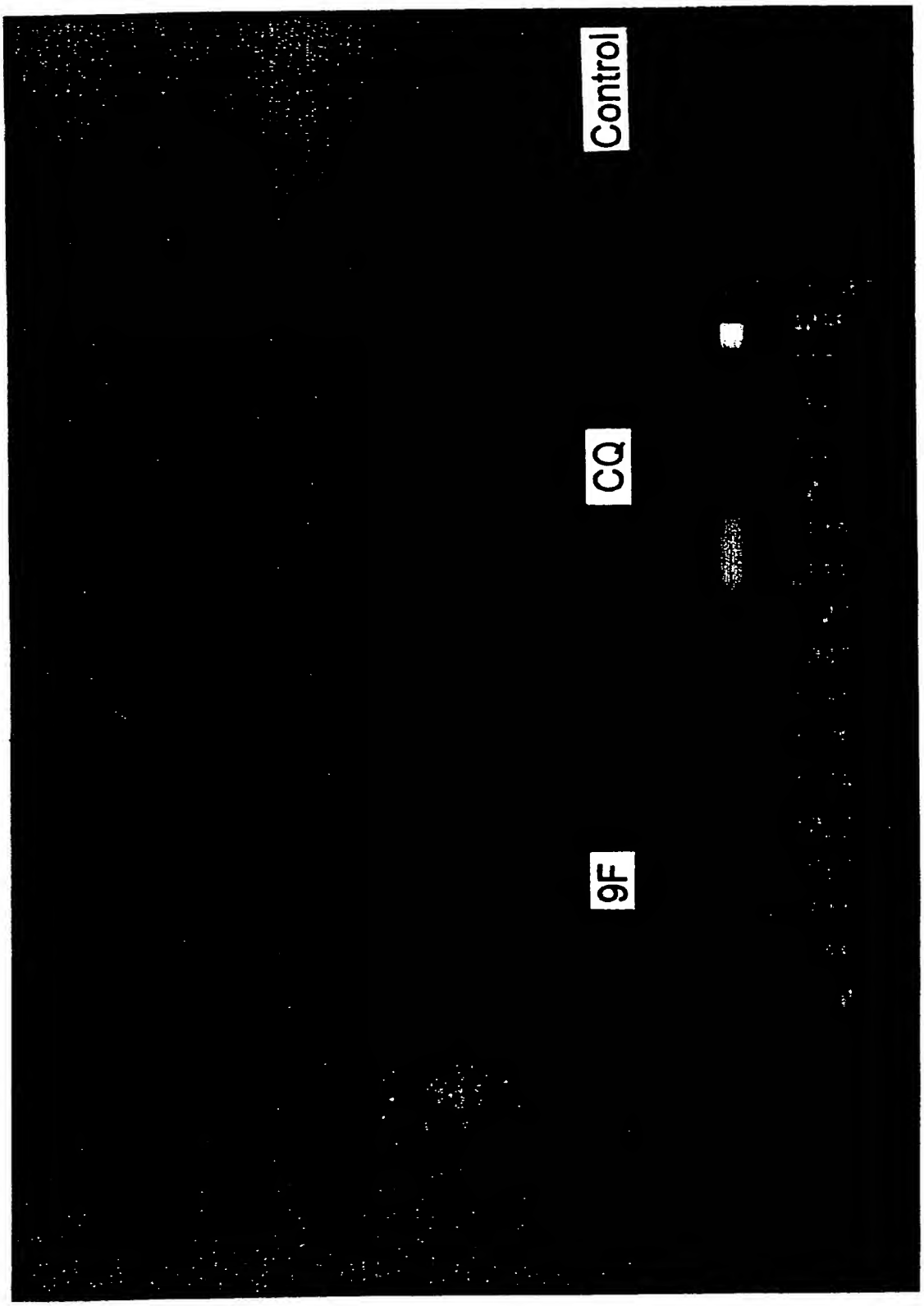

Fig. 1. Typical two-dimensional image scans (in pseudocolour) of reactive oxygen in single human submandibular-gland cells treated with photosensitizer (1 mM 9F or 1 mM CQ) and DM and irradiation with visible light for 3 min. Control cell was incubated without photosensitizer and was not irradiated.

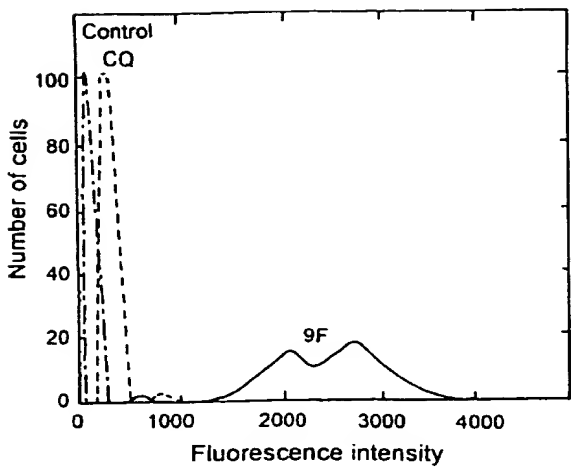

Fig. 2. Frequency distribution of the fluorescence intensity indicating reactive oxygen in single submandibular cells without or with irradiation (3 min) and photosensitizer (1 mM 9F, CQ). Line histograms were prepared with the ACAS 570 cytometer program, using 101 single cells for the 9F system ( ), 109 for the CQ (.....), and 103 for the control (----).

(10^{-7} M), a typical free-radical scavenger. On the other hand, 1 mM CQ did not significantly affect the percentage cell survival with or without the 3-min irradiaton (93% and 97%, respectively). Although photosensitizer in the presence of reducing agent alone had cell-damaging effects, which were derived from its chemical toxicological properties, the irradiation of both components with light resulted in much stronger effects, i.e. phototoxicity via free radicals, especially in the case of 9F; the percentage decrease in cell survival by irradiation of 9F (39%) was about 10-fold that of CQ (4%).

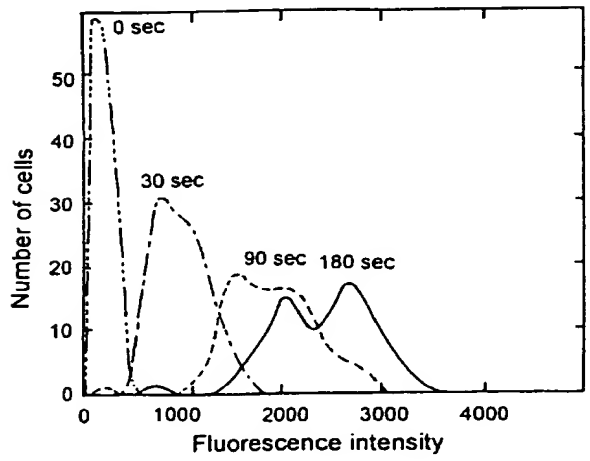

Fig. 3. Effects of irradiation time on amount of reactive oxygen generated in submandibular cells treated with the 9F system. Line histogram preparation as in Fig. 2, except that 1 mM 9F was added to the culture and irradiation with visible light was for various lengths of time: 0 sec (- ·--), 30 sec (·---), 90 sec (---), and 180 sec ( ). Line histograms for the fluorescence intensity of reactive oxygen were prepared from 104 single cells for 0 sec, 112 for 30 sec, 106 for 90 sec, and 101 for 180 sec.

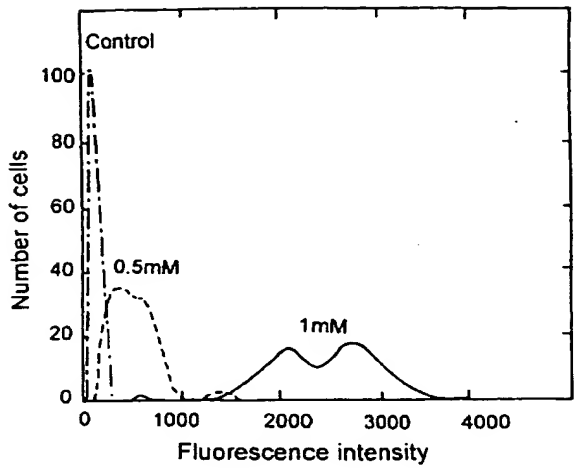

Fig. 4. Effects of concentration of photosensitizer, 9F, on reactive oxygen generated in the submandibular cells treated with the 9F system. Line histogram preparation as in Fig. 2, except various concentrations [0 mM(·), 0.5 mM (.....), 1 mM ( )] of 9F were added to the cell cultures. Irradiation with visible light for 3min. Line histograms for the fluorescence intensity of reactive oxygen were prepared from 103 single cells for 0 mM, 104 for 0.5 mM, and 101 for 1 mM.

Figure 6 shows the effects of irradiation time and concentration of photosensitizer (9F or CQ) on cell survival. With the addition of 1 mM photosensitizer, the survival decreased with time of irradiation, reaching 34% (9F) or 94% (CQ) for 5 min of irradiation. Likewise, the survival of the 3-min irradiated cells decreased with the concentration of the photosensitizer, dropping to 35% (9F) or 93% (CQ) at 1.25 mM.

Table 1
Suppressive effects of some reactive-oxygen-species scavengers on the production of reactive oxygen generated by the 9F (1 mM-3 min) system.

Scavenger	Fluorescence intensity	%Control
Control (none)	2740 ± 295	100.0 ± 10.8
Histidine (25 mM)	2162 ± 403	78.9 ± 14.7
DMSO (150 mM)	1796 ± 444	65.6 ± 16.2
SOD (300 U/ml)	2439 ± 276	89.0 ± 10.1
Catalase (300 U/ml)	1504 ± 384	54.9 ± 14.0

Cell culture with the 9F (1 m M-3 min) system was carried out as in Fig. 1, except that scavenger were added to cells 1 min before light irradiation. Data are presented as the mean ± SEM (n = 20). DMSO, dimethyl sulphoxide; SOD, superoxide dismutase.

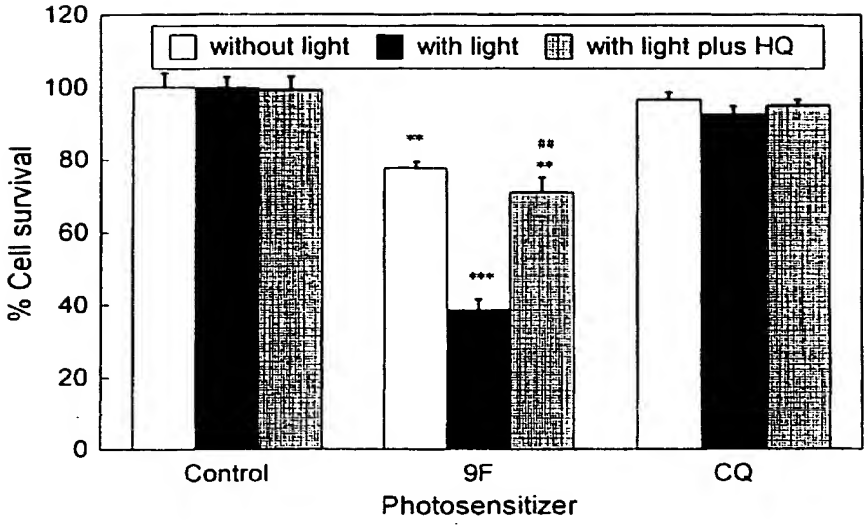

Fig. 5. Percentage of cell survival by visible light-irradiated photosensitizer (9F or CQ) in the presence of DM, and effects of hydroquinone. 9F or CQ were added at 1 mM to the wells in the presence of 1 mM DM, the cells incubated for 30 min at 37°C and then irradiated for 3 min with visible light. Number of living cells in the well was measured at 4 hr after irradiation by trypan-blue exclusion. Percentage cell survival was calculated as the ratio of the number of living cells in the experimental wells to that in the control (neither photosensitizer nor light) wells. Hydroquinone (10^{-3} M) was added to some wells at the same time that the photosensitizer was added. Data are presented as the mean ± SEM ($n = 4$). Symbols indicate a significant difference from the control culture, which was subjected to neither photosensitizer nor irradiation (**$p < 0.01$, ***$p < 0.001$) or from the irradiated 9F (without hydroquinone) culture (##$p < 0.01$).

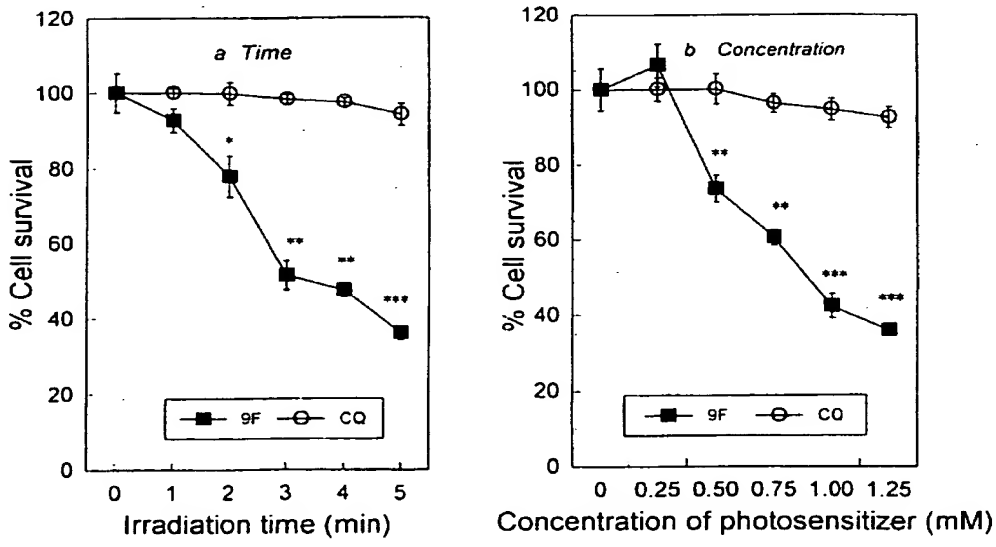

Fig. 6. Effects of irradiation time with visible light and concentration of photosensitizer on submandibular-cell survival. Photosensitizer (9F or CQ) was added at 1 mM to each well in the presence of 1 mM DM, then (a) the cells were irradiated for various times (0, 1, 2, 3, 4 and 5 min) or (b) various concentrations (0, 0.25, 0.5, 0.75, 1, and 1.25 mM) of photosensitizer (9F or CQ) were added, with irradiation for 3 min. Number of living cells in each well was measured at 4 hr after irradiation. Percentage cell survival was calculated as the ratio of the number of living cells in the experimental wells to that in the control (a; 0 min, b; 0 mM) wells. Data are presented as the mean ± SEM ($n = 4$). Symbols indicate a significant difference from the control culture (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

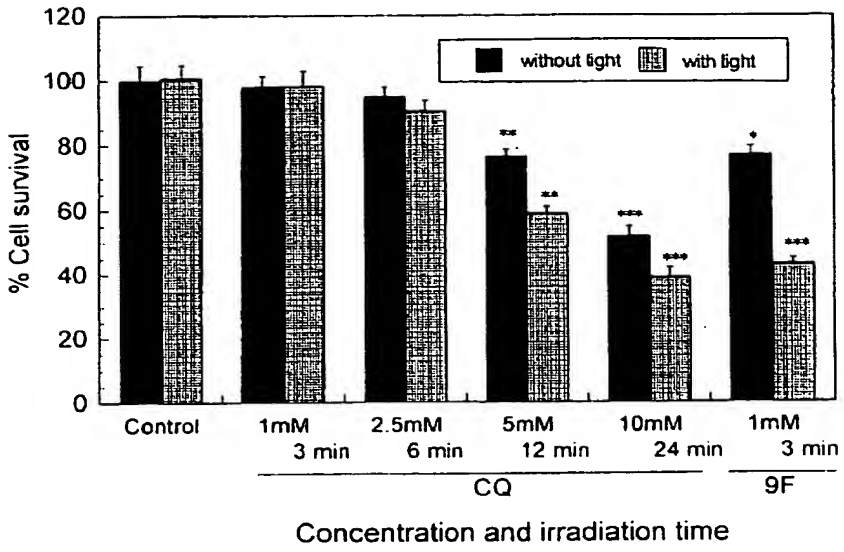

Fig. 7. Effects of higher concentrations of CQ with longer times of irradiation on percentage cell survival. The effects of various CQ concentrations (0, 1, 2.5, 5, and 10 mM) and various times (3, 6, 12 and 24 min) of irradiation were compared with the effect of 1 mM 9F and 3-min irradiation percentage cell survival. Data are presented as the mean ± SEM ($n = 4$). Symbols indicate a significant difference from the control culture (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Thus, irradiated photosensitizer-induced cell damage resulting in loss of viability was both dose- and time-dependent. The decreasing proportion of cell survival (Fig. 6) in our system paralleled the degree of production of reactive oxygen (Figs 3 and 4).

As judged from Figs 2 and 5, the cell survival was much greater and the production of reactive oxygen was much weaker in the CQ system than in the 9F. To determine if CQ was entirely lacking cytotoxicity, we measured the survival of submandibular cells treated with higher concentration of CQ and for longer times of irradiation and compared the results with those for 9F (1 mM–3 min) (Fig. 7). The percentage cell survival with 10 mM CQ and 24-min irradiation was 38%, lower than the 44% for 9F (1 mM–3 min). It was concluded that the difference in proportion of cell survival in the CQ system between with and without light was little compared with the 9F system, indicating that the photocytotoxicity of the CQ system was small even at the high concentration of 10 mM.

4. Discussion

In terms of cell survival, treatment with 1 mM 9F plus DM alone was about 80% of the value for the control culture (neither photosensitizer nor irradiaton); but, when the system was exposed to visible light for only 3 min, cell survival was decreased to about 40% (Fig. 5). The cell-damaging effects with 9F and CQ occurred in a dose- and time-dependent fashion (Figs 5 and 6). Findings for both systems were similar, but with CQ the damaging activity was much smaller than with 9F (Figs 5 and 6). Thus 9F seemed to possess powerful photocytotoxicity that was suppressed by hydroquinone. To confirm the involvement of free radicals and reactive oxygen, we sought to detect reactive oxygen in intact cells, usually this is difficult, due to the existence of endogenous scavenging agents and its short half-life (Janzen, 1984). We succeeded in detecting reactive oxygen using an ACAS laser cytometer and a peroxide indicator. The amount of reactive oxygen induced paralleled the decreasing degree of cell survival. Both were dependent on the concentration of photosensitizer and irradiation time (Figs 3, 4 and 6). The ratios of the values for the 9F and CQ systems for the decreasing degree of cell survival (Fig. 5) and for reactive oxygen production (Fig. 2) were about 10 and 9, respectively. These ratios were thus almost in agreement, indicating that the cell-damaging effects of the irradiated photosensitizer may be attributed to free radicals including reactive oxygen generated by the irradiated photosensitizer.

Although not shown in this paper, we measured the degree of cell survival with time after visible-light exposure in both systems; the observed survival did not change for 1 hr, but then decreased gradually as time after light exposure increased, suggesting that the living cells themselves had the ability to recover from free-radical damage. Free radicals induced from photosensitizers probably attack not only the double bonds of dimethacrylate monomers in resin materials but also

those of both unsaturated fatty acids and phospholipids in living cellular membranes. Furthermore, the attack on phospholipids causes cell damage due to lipid peroxidation in membranes (Terakado et al. 1984). Many researchers have demonstrated that cell damage mediated by free radicals, particularly reactive oxygen, affects virtually all cell membranes, i.e. mitochondorial, lysosomal, endoplasmic reticulum, and nuclear (Moan et al., 1989). Also, there are many radical-scavenging substances in living systems (Sies, 1993).

Cell damage in the 9F system was markedly more pronounced than in the CQ system. This ability to cause damage may be attributed to the differences in their chemical structure: 9F is an aromatic ketone with higher hydrophobicity than the aliphatic CQ. We previously reported that the haemolytic activity and the peroxidation of membranes by irradiation in the 9F system were greater than those in the CQ, based on the experiments using erythrocytes (Fujisawa et al., 1986) and phospholipid liposomes as model biomembranes (Fujisawa et al., 1986). In spite of the fact that the cell survival in the 9F (1 mM 3 min) and CQ systems (10 mM 24 min) was almost equal, the morphological changes induced in living cells by each system were not similar (data not shown), further suggesting a difference in cell-damaging effect between 9F and CQ. Furthermore, despite the fact that the 9F system produced a greater amount of free radicals in our ACAS study, the degree of polymerization of dimethacrylates by 9F was reported to be markedly smaller than that by CQ in a air atmosphere (Kadoma et al., 1986). This finding indicates that free radicals in the 9F system may be more easily changed to reactive oxygen in living cellular membranes. This remarkably strong cell damage caused by 9F suggests its possible use as a drug for photodynamic therapy, like haematoporphyrin (Dougherty et al., 1978; Henderson and Dougherty, 1992).

CQ is used generally as an initiating agent in the formation of light-activated glass polyalkenoate and resin systems such as are employed in restorative materials and denture bases (Bourke et al., 1994). The photodamaging effects of CQ were weaker than those of 9F, but those of CQ increased as its concentration and exposure time were increased (Fig. 7). However, this cell-damaging condition may be beyond the clinical level. Our data for CQ were not impressive in terms of cell-damaging effects either in the presence or absence of visible light. This supports an earlier conclusion that CQ is not classifiable as toxic, even though irradiated CQ was not studied (Hanks et al., 1991).

The reaction process of photodamage may involve type 1, via superoxide anion, or type 2, via singlet oxygen (Athar et al., 1989). In our study, the morphological changes in the submandibular cells (not shown) elicited by the 9F system resembled the nuclear burst and balloon production caused by singlet oxygen generated by rose Bengal treatment (Allison et al., 1996), which would indicate that the cell damage was caused by singlet oxygen. From the results of the scavenger test (Table 1), the 9F system seemed to generate not only singlet oxygen but also other reactive oxygen species such as hydroxy radical, superoxide anion, and hydrogen peroxide. In particular, hydroquinone, which is an inhibitor of polymerization of methacrylates, was an efficient free-radical scavenger for the 9F system.

We used cells from human submandibular gland for this study because they tended to form a uniform monolayer and would thus probably give more reproducible results than those obtained from a fibroblast cell line, for reproducible photodamage effects are dependent on equal intensity of illumination of biological targets. We are now examining the effect of photosensitizers on human gingival fibroblasts for a comparison with their effect on submandibular cells.

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Cytotoxicity of photosensitizers camphorquinone and 9-fluorenone with visible light irradiation on a human submandibular-duct cell line *in vitro*

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Abstract

The cytotoxic effect of two types of photosensitizers (camphorquinone, CQ, a widely used aliphatic type and 9-fluorenone, 9F, an aromatic type) in the presence of 2-dimethylaminoethyl methacrylate (DM) as a reducing agent with exposure to visible light (350-550 nm) was examined in a human cell line. Cytotoxicity was evaluated in terms of the percentage of cell survival, and the production of reactive oxygen in living single cells was measured with an adherent cell analysis and sorting laser cytometer and a peroxide indicator. The amount of reactive oxygen generated in the cells irradiated in the 9F (1 mM-3 min) system was about 9-fold greater than under the same conditions in the CQ system. Similarly, the decrease in cell survival in the 9F system was about 10-fold greater than in the CQ. Both the production of reactive oxygen in the cells and the decrease in cell survival paralleled the concentration of photosensitizers and the irradiation time. Although the cell-damaging effects with the CQ system were mild, at a higher dose (10 mM) and longer irradiation time (24 min) it produced cell survival equal to that in the 9F (1 mM-3 min) system. These results suggest that in the case of irradiated photosensitizer systems, 9F was much more damaging to the cells than CQ, which damage probably occurred via free radicals involving reactive oxygen generation.

Author Keywords: photosensitizer; camphorquinone; 9-fluorenone; cytotoxicity; reactive oxygen; phototoxicity

Abbreviations: ACAS, adherent cell analysis and sorting; CQ, camphorquinone; DM, 2-dimethyl-aminoethyl methacrylate; 9F, 9-fluorenone; HBS, Hepes-buffered saline

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